MODULATION OF HOST IRON COMPARTMENTS CRITICAL TO THE MALARIA PARASITE DEVELOPMENT

by

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ABSTRACT

The overlap between of malaria and iron deficiency makes it difficult to treat both conditions since iron supplementation can increase malaria and malaria decreases iron absorption. Iron chelators have proven antiparasitic activity. We characterized the antimalarial pharmacodynamics of the novel iron chelator FBS0701 in *Plasmodium* and evaluated the effect of the type and timing of iron diet on murine malaria during iron repletion. FBS0701, (S)3''-(HO)-**desazadesferrithiocin-polyether [DADFT-PE]**, is an oral iron chelator to treat transfusional iron overload. We showed that FBS0701 enter the erythrocytes and removes intracellular iron. FBS0701 reduced hepatic parasite load. FBS0701 interfered with artemisinin but was additive with chloroquine or quinine inhibition in the blood stage and with primaquine in the hepatic stage. FBS0701 killed early and late stage *P. falciparum* gametocytes. A single oral dose one day after infection cured *P. yoelii* 17XL infected mice. We studied the importance of iron compartmentalization on hepatic malaria infection on transgenic anemic mice: mice overexpressing hepcidin with reduced iron stores in the liver and hemoglobin deficient mice with normal hepatic iron stores. We found lower parasites on mice overexpressing hepcidin compared to non-anemic control mice (with normal liver iron). Parasite loads on hemoglobin deficient mice were not significantly different to non-anemic control mice. Finally, we studied the effect of low and high iron diets on the hepatic stage of murine malaria and the interplay of hepcidin with the hepatic infection levels. We used mice that were both anemic and iron deficient as well as non-anemic mice and gave both groups low and high iron diets for 2 and 6 weeks. Our results showed that high iron diets increased liver stage parasites in non-anemic mice and that iron supplementation

predominated over a negative hepcidin effect. We partially replicated in mice the human findings from the Pemba study. In conclusion, FBS0701 could be useful as malarial prophylactic or in combination with other antimalarials. FBS0701 has the potential to be used a transmission blocking agent. Increasing liver stage may synergize with blood stage increases after iron supplementation. Therefore, iron supplementation programs in malaria endemic areas, should be accompanied of antimalarial treatment.

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CHAPTER 1

INTRODUCTION: MALARIA AND THE IMPORTANCE OF IRON

1.1 Epidemiology

Over a billion people have nutritional iron deficiency worldwide. Africa and Southeast Asia account for nearly 70% of both global mortality and disability adjusted life years lost pe r year. There is also significant overlap between regions of malaria infection and nutritional iron deficiency manifested by anemia (Figures 1.1, 1.2 and 1.3).

Malaria is endemic in tropical subtropical regions, affecting especially developing countries. Approximately 3 billion people are at risk of infection and more than 240 million progress yearly towards symptomatic malaria[1]. From 2000 to 2010 there was a reduction in the number of cases by 85% in 34 of the countries that are constant fighters to eliminate malaria. More than 80% of deaths occur in Sub-Saharan Africa[2,3]. *P. falciparum* is responsible for most of the burden of disease and then *P. vivax*. *P. falciparum* is predominant in Africa, New Guinea and Hispaniola whereas *P. vivax* is predominant in the Americas and Western Pacific[3,4]. Both species are equally present in India, eastern Asia and Oceania. *P. malariae* and *P. ovale* are both uncommon, with *P. malariae* found worldwide and *P. ovale* principally in Africa.

Malaria epidemiology varies within small geographic areas. According to parasitemia rates or spleen rates in children between 2 and 9 years old, the epidemiology is classified as hypoendemic (< 10%), mesoendemic (11 – 50%), hyperendemic (51-74%) and holoendemic (> 75%). Malaria transmission is directly proportional to the vector density, the number of human bites per day per mosquito and the probability of the mosquito survival for one day[5].

In addition to the overlap between malaria and nutritional iron deficiency, malaria as a chronic infectious disease, can further decrease iron uptake from the intestine, contribute to restricted erythropoiesis, sequester bioavailable iron in ferritin stores and induce lysis of infected and uninfected red blood cells[6]. Distinguishing malaria attributable anemia from iron deficiency anemia is difficult as both overlap in host responses.

Figure 1.1. Map of Malaria-endemic countries in the Western Hemisphere [7]

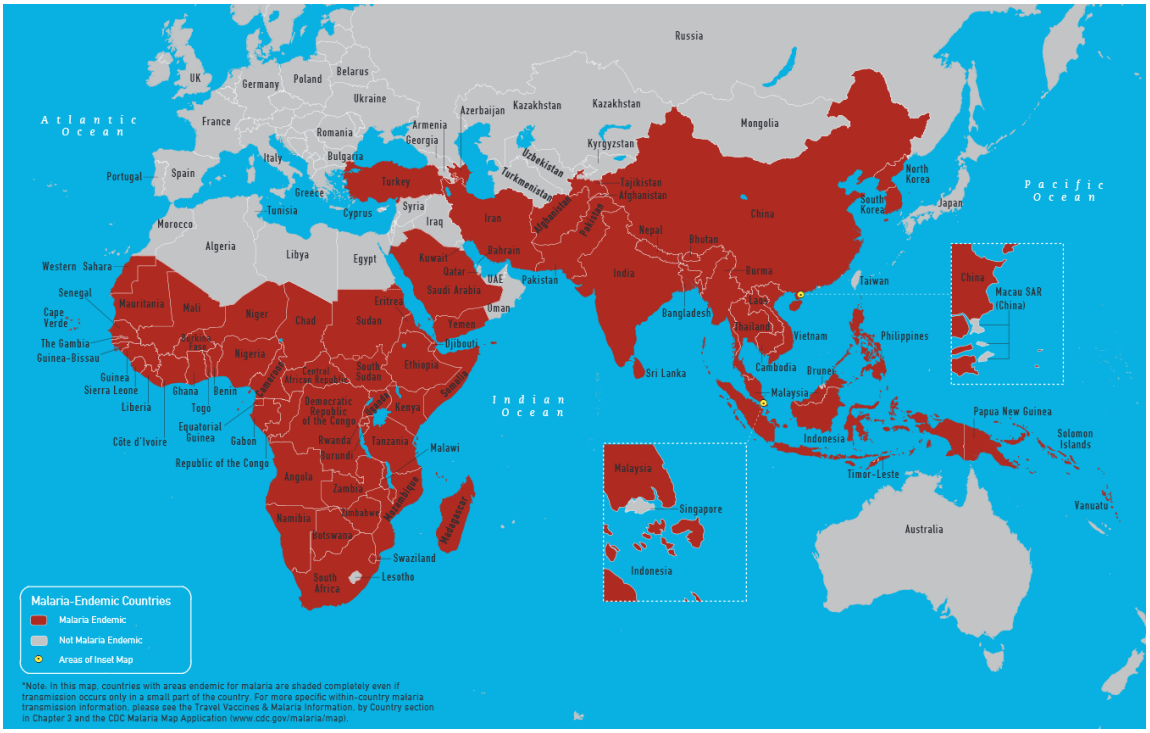


Figure 1.2 Map of Malaria-endemic countries in the Eastern Hemisphere[7]

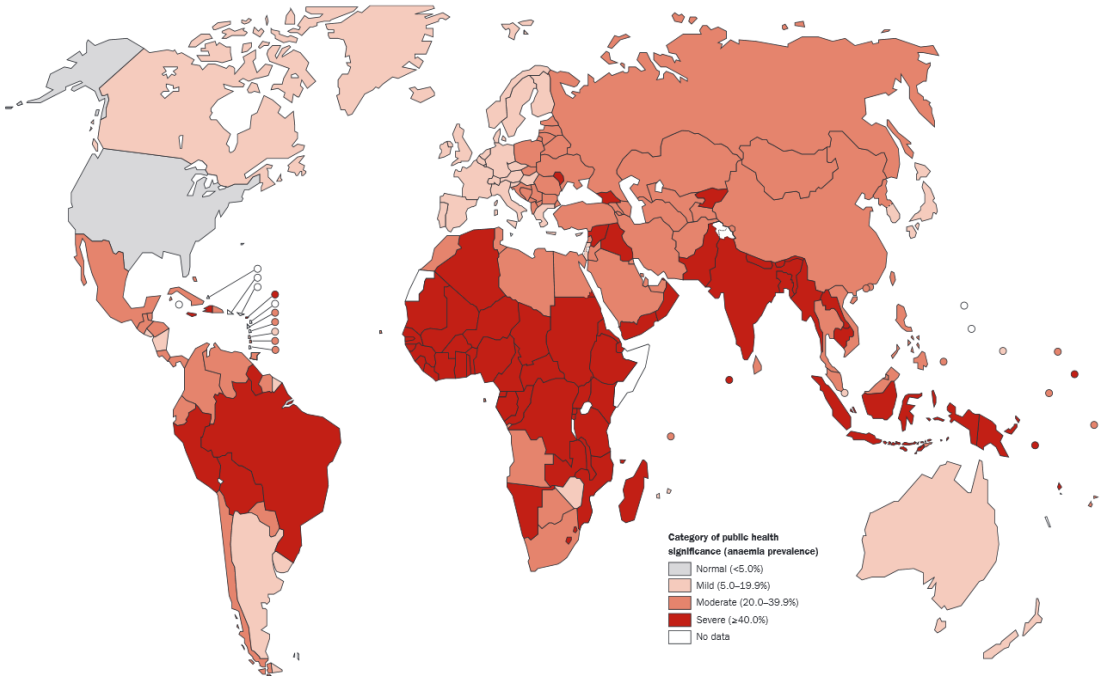


Figure 1.3. Anemia as a public health problem by country: Preschool-age children[8]

1.2 Etiology and Pathogenesis

In general, the *Plasmodium* parasite progresses from mosquito stage, to hepatic stage and finally to blood stage. This last stage contributes to anemia.

Typically infection begins with the bite to a person by a *Plasmodium* spp. infected female *Anopheles* mosquito. Humans can be infected with *P. falciparum, P. vivax, P. ovale and P. malariae*. Incubation times vary depending on the species. Parasites in the form of sporozoites enter the bloodstream and reach the liver where they multiply asexually. Both hepcidin and iron concentration modulate the number of parasites in the liver[9]. Merozoites emerge from hepatocytes and enter the bloodstream invading and multiplying in red blood cells[10].

P. vivax and *P. ovale* have a hypnozoite form and the parasite can linger in the liver for months before inducing relapse after initial infection. During erythrocytic asexual multiplication a few merozoites will develop into gametocytes (immature sexual form). When the mosquito bites, it ingest gametocytes that develop into gametes (mature sexual forms) and then into ookinetes that will cross the midgut wall of the *Anopheles*. Oocysts will rupture and release sporozoites that will reach the salivary glands. The infection cycle begins again with a new bite to another person.

The erythrocytic stage is responsible for the symptoms of malaria clinical disease. Classically malaria is the triad of fever, anemia and splenomegaly. Other symptoms of malaria include headache, fever, muscle aches, anorexia, chills, thrombocytopenia, vomiting, rigors, diarrhea, cough, hypoglycemia, retinopathy and coma in cerebral

malaria[10]. *P. falciparum* can cause severe or fatal disease by inducing the formation of knobs containing the protein PfEMP-1 on the erythrocytic membrane, which binds to receptors on the endothelial cells in post-capillary venules. The cytoadherence and sequestration of erythrocytes and the formation of rosettes through the sticking of infected and uninfected cells causes obstruction of the microvessels[11]. Sequestration in the vasculature allows parasites to reach a high biomass in the host. [12]

Plasmodium triggers anemia by: lysis of infected erythrocytes, increased clearance of uninfected erythrocytes by the spleen; and suppressing hematopoiesis after the release of cytokines caused by erythrocytic lysis or hemozoin[13].

1.3 Iron importance for living organisms

Due to its oxidation states and transition metal characteristics, iron is important in metabolic reactions in all cells, including pathogens such viruses, bacteria, fungi and protozoa[14,15]. Iron is essential for hematopoesis, oxygen delivery, neurological function, immune function and cognitive and physical development[16]. Proteins that incorporate iron include hemoglobin, myoglobin, catalase, cytochromes and iron flavoproteins like xanthine oxidase, succinate dehydrogenase and NAD dehydrogenase[17]. The metal is also important for function of organelles such as the mitochondria and chloroplast, for electron transport, iron sulfur clusters and half of the enzymes of the tricarboxylic acid cycle that bind iron[18].

Iron is rarely found as a free cation, as it can produce oxygen radicals that damage membranes and proteins. In the cells, iron is stored and transported through proteins that are regulated to ensure iron balance. Some of these proteins are ferritin, transferrin, aconitases, heme synthesis enzymes, iron transporters like the divalent metal-ion transporter (DMT) or ferroportin[17]. These proteins have a post translational regulation.

1.3.1 Iron homeostasis in humans

Human hemoglobin contains around 1700 mg of iron in adult females and 2400 mg of iron in adult males. Macrophages of the spleen and liver have around 600 mg of iron in ferritin and hemosiderin, the bone marrow around 300 mg and the liver parenchyma 1000 mg and muscles and other cells around 400 mg. The labile pool bound to transferrin in the plasma is around 3-4 mg. Bioavailable iron for daily erythropoiesis is approximately 20 mg. Iron from diet accounts for 10-15 mg of which 1 mg is absorbed and 1 mg is lost in exfoliation of cells of the skin and gastrointestinal tract[18]. There is no known mechanism for regulated iron excretion.

1.3.2 Iron physiology

The absorption of iron into enterocytes is carried out by DMT-1[19]. Iron bound to transferrin in the plasma enters to cells through transferrin receptor. Iron is exported to the plasma through ferroportin present in duodenal enterocytes, macrophages, hepatocytes, astrocytes and syncytiotrophoblasts[17]. This export is facilitated by the

multicopper feroxidases, hephaestin or ceruloplasmin[20]. Cells need iron basically for DNA synthesis and mitochondrial function.

1.3.3 Iron deficiency

Iron deficiency affects around 500 million individuals, mainly half of children and pregnant women worldwide[21]. Low iron can be caused by poor dietary intake, chronic blood loss, transfer of iron to fetus in pregnancy and hemoglubinuria[18]. Iron deficiency progresses from low iron in plasma and low transferrin saturation without anemia to low levels of hemoglobin and hypochromic and microcytic anemia. Cellular ferritin diminishes but soluble and surface transferrin receptors and zinc protoporphyrin IX increase[22]. The body slowly decreases iron in hemoglobin to preserve bioavailable iron for brain function.

1.4 Iron metabolism in the *Plasmodium* parasite

Plasmodium has an absolute requirement for iron. Once inside the red blood cell, *Plasmodium* has to get rid of the excess heme iron from hemoglobin degradation and obtain iron for its own metabolism[18]. At the erythrocytic stage, the parasite ingests 60-80% of the 5 mM hemoglobin store of the erythrocyte for its amino acid supply[23]. Because the parasite does not have a functional heme oxygenase or iron storage proteins, it handles reactive heme iron by sequestering the toxic heme, capable of oxygen radical production by the Fenton reaction, to the chemically oxidation/reduction inert hemozoin

crystal inside an oxygen-rich vacuole[23]. Interestingly in parallel the parasite syntheses its own heme[24]. This heme synthesis is not required for erythrocyte stages but is essential for mosquito or liver stages without access to hemoglobin. The asexual stage undergoes a rapid proliferation - replication of DNA and therefore requires the activity of the iron requiring ribonucleotide reductase for synthesis of nucleotides[25]. Although being in an environment with 20 mM heme iron inside the erythrocyte, iron chelators at low concentrations can decrease the development of the parasite. The amount of iron bioavailable for the parasite is low and key for its survival[25].

1.4.1 Iron sources for *Plasmodium*

Iron is needed for DNA synthesis, glycolysis, pyrimidine synthesis, heme synthesis and electron transport[18]. Chelation could potentially target the following: extracellular iron from transferrin or free iron in the media, intracellular iron bound to low molecular weight proteins, heme iron or erythrocyte ferritin iron[25]. Studies excluded the use of transferrin for iron uptake[26,27]. Removal of free iron by dialysis below 1 μM[28] or the use of impermeable dextran-DFO did not have an effect on parasite growth[29]. The erythrocytic labile iron pool was identified by calcein and was also found to be calcein labile in the parasite. Studies also showed that uninfected erythrocytes had higher total amount of labile iron pool compared to infected cells[30]. Erythrocytic ferritin can store up to 4500 iron molecules[31]. It was thought that the erythrocyte ferritin could go to the digestive vacuole along with hemoglobin and be digested by proteases releasing iron. Studies proved that iron is transported across the plasma membrane directly but not

through the digestive vacuole or deriving from heme[18]. In addition, the parasite sequence database does not report orthologs to ferroportin, ferritin, methallothione, ferroxamine-based transport systems, ferredoxin or bacterial iron siderophores[32].

1. 5 Hepcidin and regulation of iron on humans

In humans iron for erythrocyte production is obtained by two ways. Daily, humans absorb from diet 1-10 mg of iron approximately and most is incorporated into heme during erythropoiesis. Macrophages phagocytize and degrade old erythrocytes carrying around 25 mg daily that return to the circulation on transferrin [33]. The cycle of iron is regulated by a hormone called hepcidin. Iron transfer from enterocytes and recycling of iron from macrophages to serum is carried by a membrane transporter called ferroportin. Hepcidin binds to ferroportin inducing the phosphorylation, ubiquination and degradation of the transporter inhibiting iron export to the systemic circulation[34]. Hepcidin-ferroportin interaction is the dominant regulator of iron homeostasis in vertebrates and in this way hepcidin is considered the master iron regulator. Hepcidin regulates iron traffic not only at the level of dietary absorption and recycling but also at intracellular iron stores[33].

Hepcidin is poorly expressed in the heart and brain and is highly expressed in the liver as a prehormone of 84 amino acids. The prehormone is cleaved by a furin-like convertase to a 25-amino acid mature peptide (amino acids 60-84), containing eight cysteine residues that form disulfide bonds[35]. Hepcidin structure is a beta hairpin. Other

less active forms such 23 peptides are formed by cleavage of a specific dipeptidase and is required for cleavage to the 20- amino acid form[36]. With each shorter form the peptide losses critical N-terminal amino acids that decrease the ability of hepcidin to bind ferroportin. Consequently, ferroportin cannot be down regulated, leading to decreased ferritin inside the cells[37]. Humans have only one copy of the hepcidin gene (*HAMP*) but mice have duplication (*Hamp 1* and *Hamp 2*). Hepcidin is present in mammals, reptiles, amphibians and fish with multiple hepcidins in the later species. In all species studied so far hepcidins conserve the eight cysteines and invariant glycine residues. Murine hepcidin 2 cannot bind ferroportin because of a change T61I in the second amino acid of the five important N-terminal ones[35].

Most hepcidins have an antimicrobial activity. They have a weak activity against some gram-positive bacteria (*Bacillus megaterium*, *Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus carnosus*), gram- negative bacteria (*Nisseria cinera*) and the yeast *Sacharomyces cerevisiae*. Hepcidin does not have activity against the gram-negative *Escherichia coli* B21 and *Psedumonas fluorescens* or the yeast *Rhodotorula rubra*[35]. Only some hepcidins can regulate iron. *Hamp 1* is the iron regulator whereas *Hamp 2* is not[35]. Elevated synthesis of hepcidin hormone induced by ongoing blood stage parasites has been shown to inhibit the murine malaria hepatic stage on a secondary infection[9].

1.5.1 Transcriptional regulation of hepcidin

Hepcidin production is regulated at transcription not at secretion[35]. Hepcidin is upregulated by IL-6, IL-1alpha and IL-1beta with inflammation. The transcription is mediated by the binding of STAT3 to the hepcidin promoter[35]. Hemojuvelin is a surface protein associated with increased hepcidin expression[38]. This protein exists as a cleaved (cleavage between amino acids D165 and P166) or uncleaved form[39]. Hemojuvelin acts as a co receptor for bone morphogenetic protein (BMP) to enhance signaling[40]. A working model on mouse indicated that iron-induced BMP6 binds to hemojuvelin and to type I and II BMP receptors[41]. Type II BMP receptor phosphorylates and activates type I BMP receptor. Activated type I phosphorylates SMADs 1, 5 and 8 which along with SMAD 4 translocate to the nucleus to induce hepcidin expression. There are 4 types of I BMP receptors: Alk1, Alk2, Alk3 and Alk6. It was characterized on mice that Alk3 is required for basal expression of hepcidin whereas Alk2 and Alk3 (both present in the hepatocytes) are required for hepcidin expression as a response to iron and BMP signaling[42]. Mouse studies also revealed that BMP2 and BMP4 can bind to hemojuvelin and interact with BMP type I receptors Alk3 and Alk6 to activate SMAD1 to finally induce hepcidin transcription[38]. BMP7 and BMP9 can also induce expression of hepcidin independently of hemojuvelin binding[43].

1.5.2 Iron

Studies of short-term or chronic iron supplementation *in vivo* reported a ten fold increase in hepcidin synthesis[44] whereas a phlebotomy (inducing iron deficiency anemia) showed a decrease in transcription[45]. Holotransferrin inhibits the release of soluble hemojuvelin, leading to an enhanced response to BMP mediated by membrane-bound hemojuvelin[35]. In response to transferrin saturation, there is an increase in hemojuvelin sensitivity to cleavage and sensing by HFE (adult hemochromatosis gene product) and TFR2 (transferrin receptor 2 gene product) in hepatocytes[46]. Diferric transferrin re-compartmentalizes TFR2 from lysosomes to recycling vesicles. HFE increases TFR2 levels independently of diferric transferrin and consequently would indirectly regulate hepcidin synthesis[47].

1.5.3 Anemia and erythropoietic drive

Anemia can affect hepcidin expression through tissue hypoxia, erythropoietin or increased erythropoiesis[35]. Erythropoiesis is associated with increased concentrations of erythropoietin and soluble transferrin receptor and inversely associated with hepcidin synthesis[48]. The hepcidin regulation mediated by anemia and erythropoietic drive is probably independent of the regulation by iron[49]. Animal studies and hepatoma cell lines have shown that when made hypoxic they have reduced levels of hepcidin. There is a relationship between oxygen transport, erythropoiesis, iron metabolism and the physiology of hypoxic response and iron availability[50]. Hypoxia can induce the

expression of furin to cleave hemojuvelin[51].Hypoxia can also upregulate directly HIF1alpha (hypoxia-inducible factor 1, alpha subunit) expression in hepatocytes[52] and HIF1alpha inhibits stimulation of hepcidin expression by IL-6. A mutated form of the Tmprss6 protease was found in microcytic anemic mouse causing an overexpression of hepcidin[53].

In summary, hepcidin is capable of integrating signals from iron in the circulation and the liver, from bone marrow requirements and from inflammation[33].

1. 6 Hepcidin, anemia and malaria

Anemia can be produced during malaria because of hemolysis, sequestration and increased phagocytosis of both infected and uninfected erythrocytes, inability to counteract erythrocyte loss with erythropoiesis and dyserythropoiesis. Hemozoin and cytokines can inhibit erythropoietin response leading to unsuppressed hepcidin synthesis[54]. Unsuppressed hepcidin in turn increases dyserythropoiesis by iron sequestration in macrophages[55]. In malaria, there is a vicious cycle where loss of red blood cells leads to increased hepcidin and insufficient iron to support erythrocyte production. From another perspective, hepcidin could work as an innate immune regulator controlling parasitemia. Pathogens need iron for growth and have different preferences for certain types of cells[56]. For example, *Plasmodium* has hepatic and erythrocytic life cycle whereas *Mycobacterium tuberculosis* resides in macrophages. Hepcidin can determine iron availability and distribution for pathogens[33].

Both the erythrocytic and hepatic stages of *Plasmodium* need iron. Decreased levels of hepcidin can cause accumulation of iron in hepatocytes but high levels of the hormone can increase iron deposition in the reticuloendothelial system and reduce iron levels in the liver[57]. Studies showed that a current blood stage infection can trigger protection from a secondary or a superinfection with *Plasmodium* by iron redistribution: more iron in the spleen than in hepatocytes. In parallel blood stage infection could induce increased hepcidin levels in the liver of infected mice[9]. Hepcidin would restrict iron for parasite growth at liver stage infection. The interaction of hepcidin – iron – parasite suggests that iron deficiency could protect from malaria whereas iron supplementation could worsen the condition[33].

1.7 Drugs and therapy

Until an effective vaccine is released to market, drug treatment of malaria is still the main strategy to decrease the burden of the disease. The problem of resistance emergence for the common drug treatments compromises their success. Antimalarial drugs can inhibit specific molecular gene targets such as the antifolates or atovaquone targeting dihydrofolate reductase or the cytochrome bc1 complex, respectively, or can target non-mutable metabolites like the quinolines inhibition of heme crystal formation or they generate parasite toxic compounds once activated by iron or heme such as the artemisinins. Iron chelation is an additional non-mutable target.

1.7.1 Antimalarial drugs inhibiting molecular targets

1.7.1.1 Inhibiting mitochondrial cytochrome b

Cytochrome b is part of the cytochrome bc1 complex (complex III) and a main component of the mitochondrial electron transport chain (ETC). The bc1 complex is involved in the pyrimidine biosynthesis and its inhibition kills the parasite[58]. In this category we find atovaquone[59] used in combination with proguanil (called Malarone), important for treatment and prophylaxis[60]. The 4(1H)-pyridones[61] and the clinical candidate ELQ-300[62], both with similar lipohilic side chains. Two novel quinolines: SL-2-25 and SL-2-64 have dual activity against the cytochrome bc1 and the PfNDH2 (type II NADH dehydrogenase)[63]. The decoquinate containing quinoline (anticoccidial) has shown a potent *in vitro* antiplasmodial activity[64]. Also, new acridinediones (WR249685 and floxacrine) and tetracyclic benzothiazepines have been reported[65].

1.7.1.2 Inhibitors of *Plasmodium* DHODH

Plasmodium needs to synthesize pyrimidines *de novo*. Pyrimidines are precursors for DNA (thymine and cytosine), RNA (uracil and cytosine), glycoprotein and phospholipid biosynthesis. The flavoenzyme L-dihydro-orotate dehydrogenase (DHODH) is found in the matrix of mitochondria and catalyzes de conversion of L-dihydroorotate to orotate and constitutes the limiting step for the biosynthesis pathway. DHOH uses the mitochondrial ubiquinone as final electron acceptor[66]. Drugs that inhibit PfDHODH are: phenylbenzamides, ureas, naphamides, triazolopyrimidines and

thiophenes[67]. The thiophene compound Genz-669178 reported a moderate oral bioavailability in rats and dogs, and good activity *in vivo* in *P. berghei* and *P. falciparum* models[63].

1.7.1.3 Inhibitors of the *Plasmodium* folate biosynthesis pathway

Inhibition of folate synthesis and tetrahydrofolic acid recycling is effective for antimalarial treatment and prophylaxis. Dihydrofolate reductase (DHFR) is a key enzyme for folate biosynthesis and catalyzes the conversion of dihydrofolate to tetrahydrofolate. In the case of *P. falciparum* the enzyme also has a thymidylate synthase activity[68]. Pyrimethamine and cycloguanil are inhibitors of DHFR. A combination of sulphadoxine, inhibitor of the dihydropteroate synthase (DHPS, necessary for folate biosynthesis) and pyrimethamine is used as an antimalarial[69]. Clinical isolates have shown quadruple mutations in the DHFR that lead to resistance to pyrimethamine[70,71,72]. New compounds WR99210 and its prodrug PS-15 are active against the mutants. A substitution in the phenyl ring of PS-15 led to JPC-2056 maintaining the antimalarial activity[63]. P-218 is a new compound effective against wildtype and quadruple mutants and has lower toxicity in mammalian cell line. P-218 is currently another clinical candidate[73].

1.7.1.4 Inhibitors of the non-mevalonate isoprenoid biosynthesis pathway

The 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DOXPR) is involved the non-mevalonate pathway isoprenoid biosynthesis[74]. An inhibitor is fosmidomycin which

also functions as an antibacterial agent. The compound inhibits the formation of isoprenoids in the apicoplast targeting the isopentenyl diphospahte (IPP) biosynthesis. This target pathway is selective since humans obtain isoprenoids from the mevalonate pathway[63]. FR-9000098 is an analogue of fosmidomycin and is more active against *P. falciparum*. Fosmidomycin, in combination with clindamicycin (antimalarial), showed modest efficacy and safety in uncomplicated malaria in adults and children[75].

1.7.2 Antimalarial drugs with a complex mode of action

1.7.2.1 Interfering with disposition of hemoglobin heme metabolism

Heme is a metabolite target that does not mutate and is used by the quinoline drug class. The first antimalarial drug reported was quinine and then in 1940s[76] was replaced by more effective synthetic drugs, chloroquine (CQ) the most important[77]. CQ has excellent efficacy and low cost but nowadays faces widespread resistance[78]. Quinine and CQ interfere with the digestion process of hemoglobin during the intraerythrocytic development of *Plasmodium*. Specifically they prevent the formation of non-toxic hemozoin crystals by binding to growing faces of heme crystals to uncouple heme dimer incorporation into crystals[79]. Analogs of CQ with modifications to the alkyl side chain, can incorporate quinoline cores and can introduce aromatic groups in the amine located in the side chain[80]. New compounds have been derived to circumvent the CQ resistance strains: pyronaridine, naphtoquine and ferroquine[63]. Amino-alcohols can also interfere with hemoglobin metabolism, examples include mefloquine (alkaloid quinine)[81] or

halofantrine. Lumefantrine, while not a true quinoline is used in combination with artemisinin (Coartem).

1.7.2.2 Endoperoxides cause various effects in *Plasmodium*

Artemisinin is considered a prodrug activated by ferrous iron present in heme or bioavailable iron after hemoglobin catabolism by the parasite after erythrocyte invasion[82]. Iron chelation interferes with its activation and subsequent parasiticidal action. Some derivatives of artemisinins include: artemether, arteether and the carboxy-containing analogues artenilate and artesunate. Dihydroartemisinin (sesquiterpene lactone) is the active metabolite of the various artemisinins and its exact mechanism of killing is still unknown. However, it is considered to have multiple targets because of its endoperoxide functionality which translates to slow acquisition of resistance[63]. Recent cases are being reported of a delayed parasite clearance in patients[83]. Artemisinin has the highest killing rates *in vitro* and *in vivo* and a favorable safety profile. During the erythrocyte stages the artemisinins have a broadest time dependence of action, longer than the quinolines[84]. In combination with other malaria drugs (artemisinin combination therapy - ACT) they have superior clinical efficacy. The gametocytocidal activity impacts transmission in addition to clinical cure as well[85]. The most widely used is artemether, given in combination with lumefantrine (Coartem)[63]. Other derivatives with a better pharmacokinetics is the 10-(alkylamino)-ART series such as artemisone and the ozonides (1,2,4-trioxolanes)[86]. From the ozonides, arterolane (OZ-277) and OZ-439 are in clinical trials[87].

1.8 Resistance to antimalarial drugs

Chloroquine, sulfadoxine/pyrimethamine (SP) and artemisinins are part of the first line treatment for their efficacy at killing *P. falciparum* infected red blood cells and for tolerability[88]. The introduction of CQ as part of the therapy saved many lives in the early 50's especially, however the emergence of CQ resistant strains from Southeast Asia to Africa complicated any effort for eradication and cure[89]. Although CQ was replaced by sulfadoxine/pyrimethamine, resistant strains to these antifolates became widespread. Also, artemisinins important in the treatment of uncomplicated and severe malaria are facing the threat of resistance manifested by a delayed clearance in treated patients in western Cambodia[12] and Thailand[90].

Drug resistance can be produced by *de novo Plasmodium* mutations selected through drug use in one person and by the transfer of the resistant alleles via mosquito transmission to others[91]. Phenotypic changes (one or multiple) can be produced by single point mutations, modifications in various loci or gene duplication[84]. Compared to atovaquone and pyrimethamine (alone) the rate of de novo mutations with the quinolines or artemisinins leading to resistance is low.

General mechanisms of drug resistance include: decrease in the active or passive uptake of the drug, conversion of drug to inactive metabolites, increasing the expression of the target or alterations in the enzyme target[91]. *Plasmodium* parasites lack the ability to degrade or enzymatically inactivate drugs. In *Plasmodium* drug targets like cytochrome b/c, DHFR or DHODH are subjected to resistance associated mutations. The quinoline

drug concentrations are decreased by import/export modulations without altering the drug target. Importantly, *Plasmodium* resistance to iron chelation has not been shown.

1. 9. Iron chelators as antimalarial drugs

Many of the iron chelators proven to inhibit parasite growth *in vitro* are natural siderophores[25]. Important physical properties of iron chelators are: the hydrophilicity/hydrophobicity balance[92], the affinity for the iron[92], the selectivity for iron against other cations[93], the selectivity for iron (III) against iron (II)[94] and the number of coordination sites, being hexadentated chelators better to form stable complexes[25]. The major mechanisms of action include: withholding of iron necessary for metabolism such as replication[95] and forming toxic complexes with iron that once inside the infected cell that produce free radical - mediated reactions[96]. Chelators are effective *in vitro* and *in vivo*, in erythrocytic and liver stages[25]. Potential target enzymes of iron chelator are: a) ribonucleotide reductase for individual nucleotide synthesis, chelation would limit the rate of DNA synthesis[97] and therefore the reproduction of the parasite in the intra-erythrocytic stage, especially at the trophozoite stage (characterized by the highest rates of metabolism and growth)[98]and b) gamma-aminolevulinate synthase or ferrochelatase, which are enzymes for *de novo* heme synthesis in the parasite. Heme acts as a prosthetic group on cytochromes that are necessary for electron transport and mitochondrial respiration and therefore survival of the parasite[25]. Inhibition of heme synthesis by iron chelators could disrupt the electron transport system.

1.9.1 Specific antimalarial iron chelators

1.9.1.1 Desferrioxamine (DFO)

DFO is the only iron chelator in clinical use in most countries. DFO is a trihydroxamic acid derived from *Streptomyces pilosus* and it is a hexandentate chelator that complexes iron 1:1. The drug has to be given parentally and continuously[99]. DFO has been included in chelation therapies for patients with iron-loading anemia. DFO is cytocidal and specific for late trophozoites and early schizonts[100]. A multi center study in Zambia showed an increased mortality of children with cerebral malaria when they received quinine in combination with DFO[101]. Higher mortality was significantly associated with hepatomegaly and slower clearance of parasites and fever whereas lower mortality with splenomegaly and faster clearance and fever. The study could not prove a beneficial effect on mortality in children with cerebral malaria when they received quinine in combination with deforaxamine[101].

1.9.1.2 N-terminal derivatives of desferroxamine

DFO alone is poorly permeable into parasitized cells but the N-derivatives with a hydrophobic moiety to DFO shows improved permeability[94]. The most hydrophobic derivative, methylanthranilic-DFO reduces parasite growth with an IC_{50} around 4 μM compared to DFO which is around 21 μM[25].

1.9.1.3 Reversed siderophores

They represent another modified version of hydroxamate-based chelators such DFO. They are synthetic hydrophobic ferrichrome siderophores[102]. These chelators inhibit all stages of parasite. *In vitro* they are cytotoxic on rings and cytostatic on trophozoites[103].

1.9.1.4 Hydroxipyridinones

They are neutral bidentate with high specificity for Fe (III). They are given orally to treat iron overload cases[104]. *In vitro* they inhibit *P. falciparum* growth in a dose-dependent manner. The representative compound of this group is called deferiprone (L1) and inhibits more than 50% of parasite growth when the drug is applied from 5 to 100μM continuously[95]. This drug was also ineffective in a clinical trial for uncomplicated malaria[105].

1.9.1.5 Acylhydrazones

They include SIH and 2-hydroxy-1-naphthylaldehyde m-fluorobenzoyl hydrazone (HNFBH) and are effective against all stages of parasite development with an IC_{50} around 24 μM and 0.21 μM respectively. They show a dose-dependent response when are applied continuously to cells[106].

1.9.1.6 Aminothiols

They include ethane-1,2-bis(N-1-amino-3-ethylbutyl-3-thiol) (BAT) and N',N',N'-tris (2-methyl-2-mercaptopropyl)1,4,7-triazacyclononane (TAT). Their IC_{50} around 7.6 for BAT and around 3.3 for TAT. They are cytotoxic to parasites especially for trophozoites and schizonts[107].

Plasmodium has an absolute requirement for iron and a lack of receptors for iron protein-carriers. This is a key vulnerability to be exploited as a drug target. The problem of increasing resistance of the parasites to current antimalarial drugs has posed the need to develop new mechanisms of antimalarial drug action which are also efficacious, safe and not prone to resistance. Iron chelation represents a new drug target to affect multiple stages of the life cycle of parasite. Iron chelation has the advantages of being a non-mutable target like enzyme targets and it avoids the problem of targeting a specific structure for activity such in the cases of hemoglobinases.

1.10 FBS0701

1.10.1 Structure

FBS0701 is a chemical analogue to bacterial siderophore desazadesferrithiocin[108]. FBS0701 is tridentate iron chelator (Figure 1.4). Like other siderophores, FBS0701 binds to Fe (III) with high affinity and specificity over Fe (II) and has an equilibrium constant of 10^{-22}M[109].

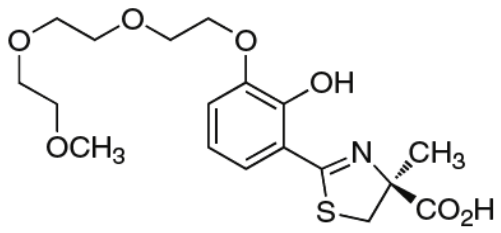


Figure 1.4. Chemical structure of FBS0701 or (S)3-(HO)-desazadesferrithiocin-polyether [DADFT-PE]. The chemical name is (S)-4,5-dihydro-2-[2-hydroxy-3-(3,6,9- trioxadecyloxy)phenyl]4-methyl-4-thiazolecarboxylic acid

1.10.2 Pharmacokinetics

In normal human volunteers and in iron overload patients orally dosed with 3-40 mg/Kg of FBS0701, the pharmacokinetics across the dosing range shows high bioavailability (>60%), dose-proportionality and a terminal $t_{1/2}$ averaging 20 hours making once daily dosing feasible[110]. The addition of the polyether sidechain eliminated nephrotoxicity[110]. The C_{max} averaged 170 μM with an $AUC_{0\text{-}24h}$ of 385 μM.hour/L at the 32/kg/d dose level (Table 1.1).

Parameter*	Dose			
	3 mg/kg/day	8 mg/kg/day	16 mg/kg/day	32 mg/kg/day
C_{max} (ng/mL)	5,910±2,298 (4)	15,000±4,439 (4)	38,225±3,947 (4)	68,250±27,519 (4)
T_{max} (h)	1.31 (4)	1.18 (4)	1.00 (4)	1.49 (4)
AUC(0-24) (h×ng/mL)	19,476±11,327 (4)	44,916±30,751 (4)	92,261±36,560 (4)	157,577±43,484 (4)
λz (h^{-1})	0.0655±0.0606 (4)	0.0424±0.0249 (4)	0.0421±0.0246 (4)	0.0381±0.0091 (2)
$t_{1/2}$ (h)	16.2±8.32 (4)	20.9±11.3 (4)	21.3±11.8 (4)	18.7±4.48 (2)
CL/F (mL/min)	162±85.0 (4)	225±142 (4)	206±78.4 (4)	172±60.1 (4)
Vz/F (L)	185±84.1 (4)	311±137 (4)	339±166 (4)	214±2.94 (2)
Ue(0-24) (mg)	66.1±24.0 (4)	201±68.1 (4)	402±103 (4)	641±208 (4)
Fe(0-24) (% Dose)	44.0±16.0 (4)	47.4±13.8 (4)	39.2±5.86 (4)	43.1±15.8 (4)
CLr (mL/min)	75.2±46.8 (4)	105±62.9 (4)	83.2±36.5 (4)	73.6±37.8 (4)

Values are reported as arithmetic mean ± standard deviation except T_{max} for which the median is reported.

Table 1.1. Pharmacokinetic parameters of FBS0701[110]

1.10.3 Safety

FBS0701 was well tolerated during a 7 day human dosing period with no dose-dependent adverse events and no serious adverse events related to the drug[110]. In another 24 week safety and efficacy study for transfusional iron overload similar results were found[111].

Based on these promising pharmacokinetic and safety data, 3 to 5 day 32 mg/kg course of treatment should be sufficient to clear parasites by maintaining mean plasma concentrations of FBS0701 well above the IC_{50} over 72-120 hour period[110]. The amount of iron excreted over this anticipated treatment period for malaria would not exceed 90-120 mg out of a total body iron of approximately 3000 mg or about 3% of iron stores transiently removed[18].

1.11 Iron interventions

One of the more recent controversial types of iron intervention is iron supplementation in malaria endemic areas. The iron supplementation policy of anemic individuals has been strongly debated since the study on Pemba Island that was hyperendemic for malaria. The study showed that iron and folate supplementation increase risk for hospitalization and death attributed (but not proven by a laboratory diagnosis) to be from malaria in iron replete but not in iron deficient children regardless of anemia[112]. From this study, programs on iron supplementation in malaria endemic areas were halted and also led medical health workers to reconsider the approach of iron

supplementation by screening out children who are iron-replete or combining supplementation with control strategies for the disease such as antimalarial treatment.

In general, iron intervention strategies are based on the target population, the local health care and/or industry infrastructure available and local dietary and cultural habits and costs. The supply of iron amount is dependent on the iron requirements of the population and if their intention is to treat or prevent iron deficiency[16]. There are four main types of intervention:

1.11.1 Dietary diversification

This strategy increases the bioavailability of iron of traditional food or the consumption of food rich in iron.

1.11.1.1 Bioavailability of iron

1.11.1.1.1 Direct manipulation of food

The processing of food and the techniques used for preparation could increase iron bioavailability of foods. Fermentation can reduce 90% of phytate that chelates iron, germination between 50-64% and soaking from 47-98%[113]. The type of strategy alone is sufficient to improve iron status. There are not studies about the impact on malaria.

1.11.1.1.2 Indirect manipulation of food

It is especially used in developing countries to reduce iron deficiency. A strategy is cooking in iron pots that can increase iron available for absorption but can also cause iron overload[114]. Also, there are not studies about the impact on malaria.

1.11.1.2 Foods rich in iron

Iron content is very high in animal foods such pork, poultry, beef and fish and in special organs like liver. Unfortunately, poor women who are at higher risk of iron deficiency cannot afford flesh foods[16].

1.11.2 Food fortification

It is referred to adding iron to food deliberately. Iron concentrations taken daily by fortification is less than taken by supplement.

Organoleptic characteristics of iron make it hard to add the nutrient to foods. Ferrous sulfate is efficacious in fortified foods and is accepted by the WHO[115]. Ferrous fumarate lacks of studies to support its efficacy[16]. NAFeEDTA has proven effective in studies improving iron status in women and children. Other alternatives as ferric pyrophosphate and electrolytic iron are more expensive.

Some methods of fortification are:

- Centrally fortified foods: distribute iron on staple foods, beverages or condiments.
- Home fortification: implies adding iron directly to meals when eating. This is mainly used for treatment than prevention.
- Biofortification: is a future strategy where iron concentration of staple food is increased by plant breeding or genetic engineering[16].

1.11.3 Iron supplementation

It is the most common method to treat anemia and iron deficiency. It consists on the oral delivery of iron in the form of a pill or liquid without the intervention of food. It is safe, efficacious and less expensive. Programs with iron supplementation have proven to be effective at prevention of iron-deficient anemia[116]. The side effects of iron supplementation are nausea, diarrhea or constipation, anorexia and dizziness. A study in Gambia showed that using between 0.5 mg and 1.5 mg of folic acid along with oral iron (47 mg) there was not interference with sulfadoxine - pyrimethamine in pregnant women[117].

Based on the statement that iron supplementation increases susceptibility to malaria, then the opposite, meaning iron-deficient and/or anemic people could have less risk to malaria. Studies have shown contradictory observations at testing this idea. Two often cited older human studies associated injectable iron or oral refeeding in famine stricken refugees with high percentage of symptomatic malaria[118,119]. Other studies, including a meta-analysis, have not shown exacerbation of human malaria with oral

replacement[120]. However, iron supplementation policy has been debated because of a study done in Pemba (hyperendemic for malaria)[112]. The study showed that iron and folate supplementation increase the risk for hospitalization and death from malaria in iron replete rather that iron deficient children regardless of anemia. Oral iron (12.5 mg) and folate (50 μg) had a protective effect on disease in children who were both iron deficient and anemic; no effect in children iron deficient and with no anemia, but had a significant risk of adverse outcomes measured in hospital admissions and deaths in children iron replete with or without anemia[112].

1.11.4 Non-dietary interventions

It is based on delaying the cord clamping by 2 – 3 minutes to have a higher transfusion of placental blood to the baby. The baby can a have a total increase in iron content at birth preventing iron deficiency during his/her 1st year. There is not specific safety and efficacy data about this method in malaria endemic areas[16].

1.12 Thesis overview and objectives

The broad goal of the project was to better understand the importance of iron compartmentalization and bioavailability of the host on *Plasmodium* development. The world faces increasing resistance of parasites to current antimalarial drugs that create a need to develop new drugs with novel mechanisms of action efficacious, safe and not prone to resistance. Considering iron as a non mutable target, we studied the

consequences of iron chelation on the blood, liver and mosquito stages of the parasite life cycle. We tested a new iron chelator known as FBS070 *in vitro* and *in vivo* mouse model.

The problem of geographical overlap between iron deficiency anemia and malaria brings controversy on how effective iron supplementation interventions are to treat anemia in malaria endemic areas. In this research, we evaluated the type and timing of iron diet during iron repletion on hepatic parasite load. On parallel, we studied the importance of the iron regulatory hormone hepcidin on infection and its interplay with the iron diets. Also, we studied liver stage infection on genotypic anemic mice receiving exogenous hepcidin and confirmed the importance of the hormone and the consequent compartmentalization of iron it induces.

Aims:

Aim 1. To characterize the antimalarial pharmacodynamics of the novel iron chelator FBS0701.

We hypothesize that FBS0701 can function as an antimalarial drug that targets the hepatic, erythrocytic and mosquito stages.

Specific aim 1.1: To identify the FBS0701 effective dose, timing and duration of antimalarial action *in vivo* and the IC_{50} and mechanism of action *in vitro*.

Specific aim 1.2: To characterize the interaction of FBS0701 with other antimalarials on blood and hepatic stages *in vitro* and *in vivo.*

Specific aim 1.3: To test the activity of FBS0701on mosquito stages previous treatment of *P. falciparum* gametocytes.

Aim 2. To evaluate the effect of the type and timing of iron diet on murine malaria infection during iron repletion with implications for human nutritional iron supplementation in malaria endemic areas.

We hypothesize that bioavailable iron for the parasite influences the hepatic stage significantly more. The type or timing of iron supplementation during iron replenishment will alter malaria outcome dependent on an iron compartmentalization effect.

Specific aim 2.1: To compare the effect of a low, normal and high iron diet given for two weeks versus six weeks on hepatic malaria infection in iron replete mice and iron deficient mice.

Specific aim 2.2: To evaluate the level of hepatic malaria infection on transgenic overexpressing hepcidin (Tg+) mice upon the addition of exogenous hepcidin and on hemoglobin deficient mice (hbd).

Specific aim 2.3: To correlate malaria hepatic outcomes with the level of the iron regulatory hormone hepcidin in transgenic anemic mice, anemic wild type inbred mice and iron replete wild type inbred mice.

CHAPTER 2

ANTIMALARIAL IRON CHELATOR, FBS0701, SHOWS ASEXUAL AND GAMETOCYTE *PLASMODIUM FALCIPARUM* ACTIVITY AND SINGLE ORAL DOSE CURE IN A MURINE MALARIA MODEL

2.1 ABSTRACT

Iron chelators for the treatment of malaria have proven therapeutic activity *in vitro* and *in vivo* in both humans and mice, but their clinical use is limited by the unsuitable absorption and pharmacokinetic properties of the few available iron chelators. FBS0701, (S)3''-(HO)-**desazadesferrithiocin-polyether [DADFT-PE]**, is an oral iron chelator currently in Phase 2 human studies for the treatment of transfusional iron overload. The drug has very favorable absorption and pharmacokinetic properties allowing for once-daily use to deplete circulating free iron with human plasma concentrations in the high μM range. Here we show that FBS0701 has inhibition concentration 50% (IC_{50}) of 6 μM for *Plasmodium falciparum* in contrast to the IC_{50} for deferiprone and deferoxamine at 15 and 30 μM respectively. In combination, FBS0701 interfered with artemisinin parasite inhibition and was additive with chloroquine or quinine parasite inhibition. FBS0701 killed early stage *P. falciparum* gametocytes. In the *P. berghei* Thompson suppression test, a single dose of 100 mg/kg reduced day three parasitemia and prolonged survival, but did not cure mice. Treatment with a single oral dose of 100 mg/kg one day after infection with 10 million lethal *P. yoelii* 17XL cured all the mice. Pretreatment of mice with a single oral dose of FBS0701 seven days or one day before resulted in the cure of some mice. Plasma exposures and other pharmacokinetics parameters in mice of the 100 mg/kg dose are similar to a 3 mg/kg dose in humans. In conclusion, FBS0701 demonstrates a single oral dose cure of the lethal *P. yoelii* model. Significantly, this effect persists after the chelator has cleared from plasma. FBS0701 was demonstrated to remove labile iron from erythrocytes as well as enter erythrocytes to

chelate iron. FBS0701 may find clinically utility as monotherapy, a malarial prophylactic or, more likely, in combination with other antimalarials.

2.2 INTRODUCTION

Iron metabolism is a proven target for many malaria drugs. The quinolines like chloroquine and quinine interfere with iron protoporphyrin IX crystallization in the digestive vacuole[121]. The artemisinins are activated by iron to generate carbon-centered radicals that rapidly kill parasites[122]. Iron chelators have been explored as alternative malaria drugs for decades[25]. Intravenous deferoxamine does increase clearance of parasites[123] but in an important clinical trial deferoxamine did not affect outcome from severe cerebral malaria[124]. The orally available deferiprone was also suboptimum in an uncomplicated malaria trial because of poor iron clearance after oral absorption[105]. Iron regulation is potent in limiting liver stage multiplication mediated, in part, by hepcidin pathway[9]. Iron supplementation increases hepatic parasites[125] and iron chelators decrease hepatic parasites[9]. For intraerythrocyte *Plasmodium*, the literature describes chelation of both labile iron pools in parasites as well as infected erythrocyte cytosol. Deferoxamine decreases available iron in the nucleus and parasite cytosol. The exact mechanism of parasite killing is not known but evidence points to a iron chelator mechanism of limiting iron for ribonucleotide reductase production of nucleotides, however earlier studies also suggest a toxic mechanism of erythrocytic *Plasmodium* killing[25]. A drawback for *Plasmodium* iron chelation chemotherapy is interference with the parasiticidal action of artemisinin drugs[126,127].

The potent novel chemical analogue of the bacterial siderophore desferrithiocin is entering clinical trials for transfusional iron overload. **(S)-4,5-dihydro-2-[2-hydroxy-3-(3,6,9-triox-adecyloxy)phenyl]4-methyl-4-thiazolecarboxylic acid** also known as (S)3''-(HO)-desazadesferrithiocin-polyether [DADFT-PE] or more simply as FBS0701 has a very high affinity and specificity for Fe (III) with an equilibrium constant of 10^{-22} Molar[109]. The molecular weight of the formulated salt is 441 while free base is 400. The iron clearing efficiency in rodents is 10.6 ± 4.4% and in primates is 23 ± 4.1%[109,128]. In contrast the iron clearing efficiency for deferoxamine is 2.8% and 5.5% and deferiprone is 1.2% and 2.1% in rodents and monkeys, respectively. In normal human volunteers dosed with FBS0701 with 6, 10 or 16 mg/kg, the pharmacokinetics across the dosing range indicates high bioavailability C_{max} of 22.7, 41.5 and 87.0 μM respectively, dose-proportionality and a terminal $t_{1/2}$ permitting once-daily dosing[110]. Here we investigated the actions of this iron chelator on *P. falciparum* blood stage parasites, its action against gametocytes and efficacy in murine malaria models.

2.3 Methods

2.3.1 Materials

FBS0701 was obtained from Aptuit (Kansas City) LLC under good manufacturing practice. All other reagents and chemicals are from Sigma unless otherwise noted. *P. falciparum* isolate 3D7, was obtained from ATCC-BEI MR4 repository.

2.3.2 *P. falciparum* culture

Direct effect of FBS7010 on *P. falciparum* was determined by SYBR green I (Invitrogen-Molecular Probes, Eugene OR) based assay as described earlier[129]. Briefly, the inhibition assay was initiated by adding serially diluted FBS7010 in 96 well microplates followed by sorbitol synchronized 1% ring stage parasites at 1% hematocrit in RPMI 1640 media with glutamine and HEPES supplemented with 10% human serum and 50 mg/ml hypoxanthine. Culture plates were then transferred to 37ºC incubator and maintained in the environment of 5% O_2, 5% CO_2 and 90% N_2 for 72 hrs. 2x SYBR Green I solution in lysis buffer was added after one cycle of freeze and thaw. Plates were then placed at room temperature in dark for 1–2 hours and fluorescence values were quantitated in a plate reader (HTS 7000, Perkin Elmer) at emission and excitation wavelengths of 535 and 485, respectively. For fractional inhibition analysis quinine, quinidine, chloroquine and artemisinin were used at concentrations ranging from 1 to 100 nM and FBS0701 from 1–15 μM. The fractional inhibition was analyzed according to the original Elion and Hitchings paper[130] with modifications by Bell[131]. The fraction of the chloroquine, quinine or artemisinin drug in combination with FBS0701 that produced an IC_{50} was divided by the IC_{50} of the chloroquine, quinine or artemisinin alone for the ratio on the y-axis. The x-axis was fraction of FBS0701 in combination with the chloroquine, quinine or artemisinin divided by concentration of FBS0701 which produced an IC_{50}.

To test the effect of FBS0701 on gametocyte stages, *P. falciparum* NF54 cultures were initiated in 24 well plates at 0.5% asexual parasitemia and 4% hematocrit[132]. Medium was changed daily up to day 18, without addition of fresh RBCs. Continuous cultivation without dilution leads to concomitant crash of asexual parasitemia and

induction of gamteocytogenesis by day 5. The gametocytemia was approximately 7% at start of treatment. FBS0701 was dosed in wells at Days 9–10, when majority of gametocytes were in early stage of development (Stage I, II) or Days 14–15 when majority of gametocytes have matured to stage III to V. Levels of gametocytemia were determined on day 18 and the mean number of gametocytes was calculated by counting 10 high-powered (1000 x) fields from triplicate wells per condition. More than 1000 erythrocytes were enumerated by random scanning across Giemsa blood film.

2.3.3 Animal ethics

All animal experiments were performed on protocol ‘‘Malaria Drug Testing in Mice’’ (Approval ID-MO09H401) approved by The Johns Hopkins Animal Care and Use Committee in accordance with institutional standards.

2.3.4 Malaria murine drug testing

Male 5–6 week old at approximately 25 ± 2 g C57/BL6 mice were purchased from Jackson Laboratories (Maine, USA). The *P .berghei* ANKA strain was obtained from ATCC; the *P. yoelii* 17x lethal strain was the gift of James Burns (Drexel University). Freshly made solutions of FBS0701 in water were used for all the experiments and drug was administered by oral cannulation at indicated times. Murine infection was initiated by intraperitoneal inoculation of 10 million infected red blood cells for *P. berghei* and 10 million for *P. yoelii*. Blood was taken from the tail vein for blood smears.

Parasitemias were determined in a blinded fashion by counting four fields of approximately 200 erythrocytes per field. Mice that survived for 30 days post infection with complete disappearance of parasitemia and no recrudescence within the next 30 days were considered cured.

2.3.5 Pharmacokinetics

30 female Balb/c mice were dosed with 100 mg/kg of FBS0701 by oral cannulation and blood was drawn by cardiac puncture during anesthesia. Whole blood was separated into plasma and erythrocytes and plasma was sent for analysis by Covance Laboratories (Madison, WI) using a validated assay[110].

2.3.6 Measurement of bioavailable labile iron in erythrocytes

Blood was collected from healthy subjects washed 2 times with PBS. Erythrocytes were then incubated with 0.125 μM calcein (Invitrogen) in PBS for 15 minutes at 37ºC, washed 2 times with PBS and then allowed to rest for 10 minutes at 37ºC in the dark[133,30,134].

Cells were incubated with PBS, 100 μM FBS0701 or 100 μM diferiprone for 1 hour at 37ºC. Cells were analyzed by flow cytometry using a modified FACS-Calibur with 2 lasers 30 mW 488 Diode Pumped Solid State laser and a 25 mW 637 red diode laser (FACS-Calibur; Becton Dickinson, Mount View CA, modified by Cytek Development). Data was collected using FlowJo CE and analyzed using Summit v4.3.01. The emission of 400,000 cells was analyzed using logarithmic amplification for fluorescence signal height (FL-1, 530/30) and linear amplification for forward scatter

(FSC) and side scatter (SSC). The threshold was set at FSC to exclude cell debris and microparticles. The mean fluorescence intensity (MFI) was calculated using Summit v4.3.01. Individual experiments were performed in triplicate and also biologic duplicate with different human erythrocyte donors. The statistical significance was calculated using the Student's t-test.

2.4 RESULTS

2.4.1 *P. falciparum* blood stage inhibition

In the chloroquine and quinine sensitive isolate 3D7, the IC_{50} with continuous drug exposure was 6 μM (IC_{50}=3 μM and IC_{90}=10 μM). Morphologically the FBS0701 exposed parasites were shrunken. Stage and concentration dependence of FBS0701 iron chelation showed that for continuous drug exposure starting at ring and trophozoite stage the IC_{50} was similar at 6 μM, but when drug exposure was initiated at schizont stage after DNA replication, the IC_{50} increased by almost three fold to 15–17 μM. Investigation of dose-dependent killing of gametocytes shows that 100 μM inhibited more than 90% of early stage I and II gametocytes but only about 40% of late stage III or IV gametocytes, 10 μM FBS0701 had minimal 37% decrease in the number of gametocytes (Figure 2.1). Because of the known interactions of iron chelators with the artemisinins[126,131,135], we investigated interaction *in vitro* with *P. falciparum*. Fractional inhibition indicates interference with artemisinin and an additive action with the quinolines like chloroquine or quinine (Figure 2.2).

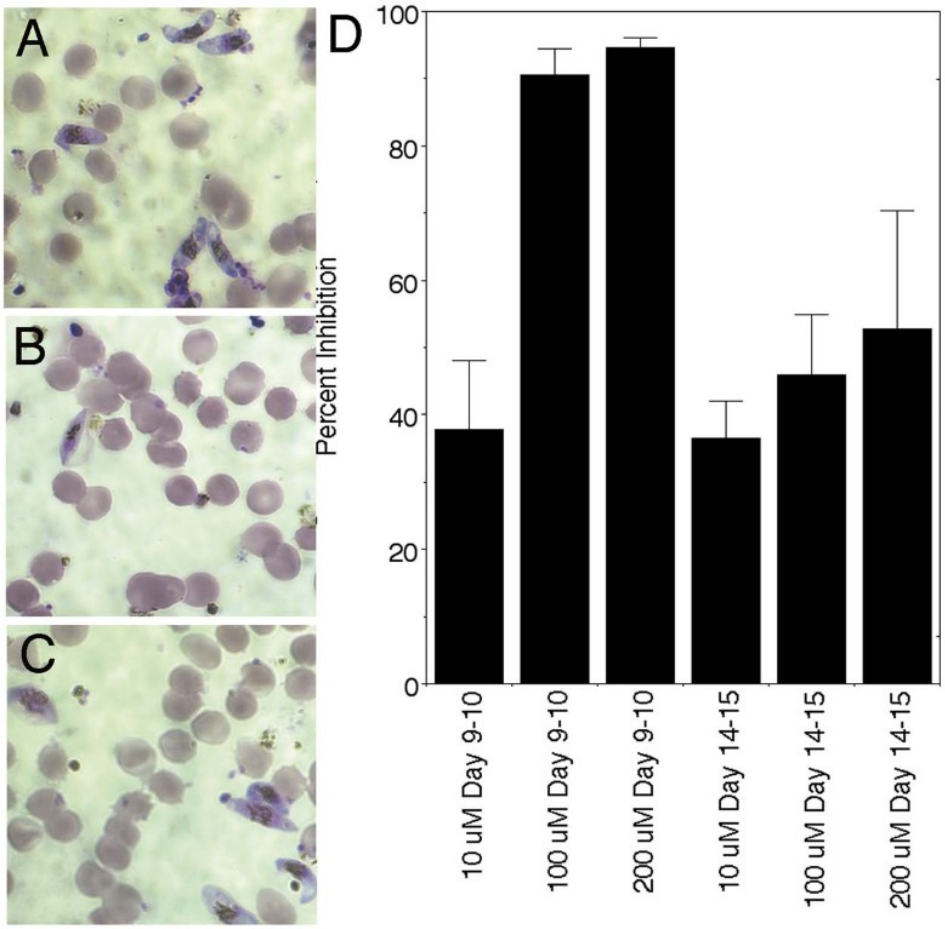


Figure 2.1. FBS0701 inhibits early stage not late stage gametocytes. Gametocyte cultures *of P. falciparum* NF54 strain were initiated at 0.5% asexual parasitemia and 4% hematocrit in 24 well culture plates. Gametocytes were morphologically determined by Giemsa stain and counted on Day 18 with no drug (**A**) or 100 μM FBS0701 dosed in wells at Days 9–10 (**B**) or Days 14–15 (**C**) post-culture initiation. Dose dependent inhibition is more pronounced with early gametocyte stage I and II (day 9–10 dosing) than gametocyte late stages III-IV (day 14–15 dosing) (**D**). Gametocytemia was approximately 7% at the start of treatment with FBS0701 on indicated days. Gametocytes per 10 high power fields (excess of 1000 erythrocytes) in each of three replicate wells for each condition were counted on Day 18. Control wells had 7.4% mean gametocytes. Data expressed as percent inhibition of control. Standard deviation of triplicate observations for each of the three wells is shown.

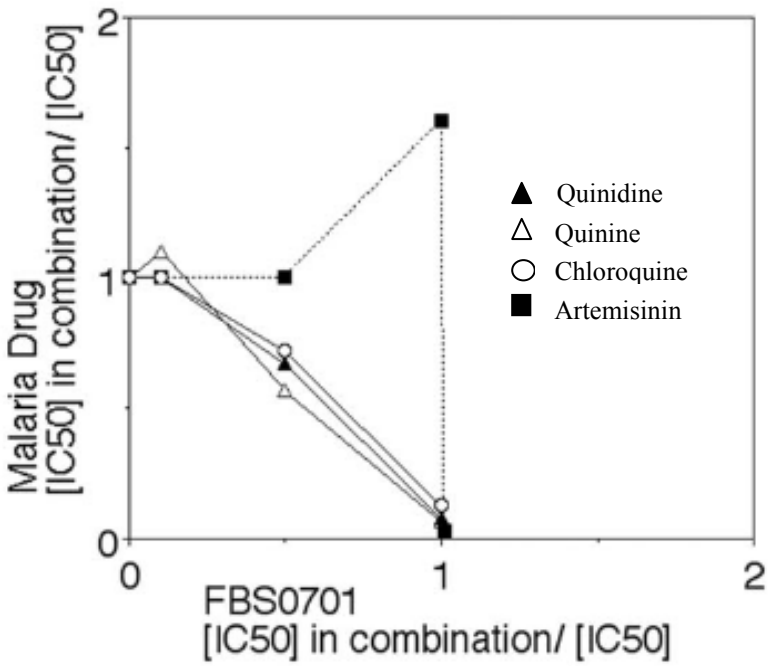


Figure 2.2. FBS0701 interferes with artemisinin but not the quinolines like chloroquine or quinine. Fractional inhibition curve for the quinolines and artemisinin with FBS0701. Up to 10 μM FBS0701 does not change the low nM IC_{50} of artemisinin (filled square). In contrast for the quinolines 1, 5, 7.5 and 10 μM FBS0701 reduced the IC_{50} in an additive manner for chloroquine (empty circle), quinine (empty triangle) or quinidine (filled triangle). For each axis the concentration of drug in combination which equals the inhibition concentration 50% was divided by IC_{50} of drug alone.

2.4.2 Murine malaria inhibition

In the lethal *P. berghei* ANKA mouse model, FBS0701 showed a 50% reduction in day 5 parasitemia and delayed death by more than ten days at the 100 mg/kg dose, regardless of single day dosing for one day, three days or seven days (Figure 2.3). The lethal *P. yoelii* model was used next because while lethal infection results from invasion of both normocytes and reticulocytes, a recent vaccine study demonstrated that by largely restricting invasion to reticulocytes the infection was no longer lethal[136].

We dosed mice with a single oral dose of FBS0701either seven days or one day prior to parasite inoculation and also dosed a day after inoculation. All infected untreated control mice died by day 11. All the mice treated a day after infection with a single oral dose of the iron chelator survived (Figure 2.4). Surprisingly two to three mice with FBS0701 pretreatment one or seven days prior to intraperitoneal *P. yoelii* infection still lived suggesting a lingering effect after the drug was cleared. In this model we again saw a small reduction in day 3 parasitemia. In all surviving mice, which had been dosed with FBS0701, the day 16 parasitemia was near 50% but confined to reticulocytes rather than normocytes (Figure 2.5). Resolution of parasitemia coincided with return of reticulocyte count to normal levels.

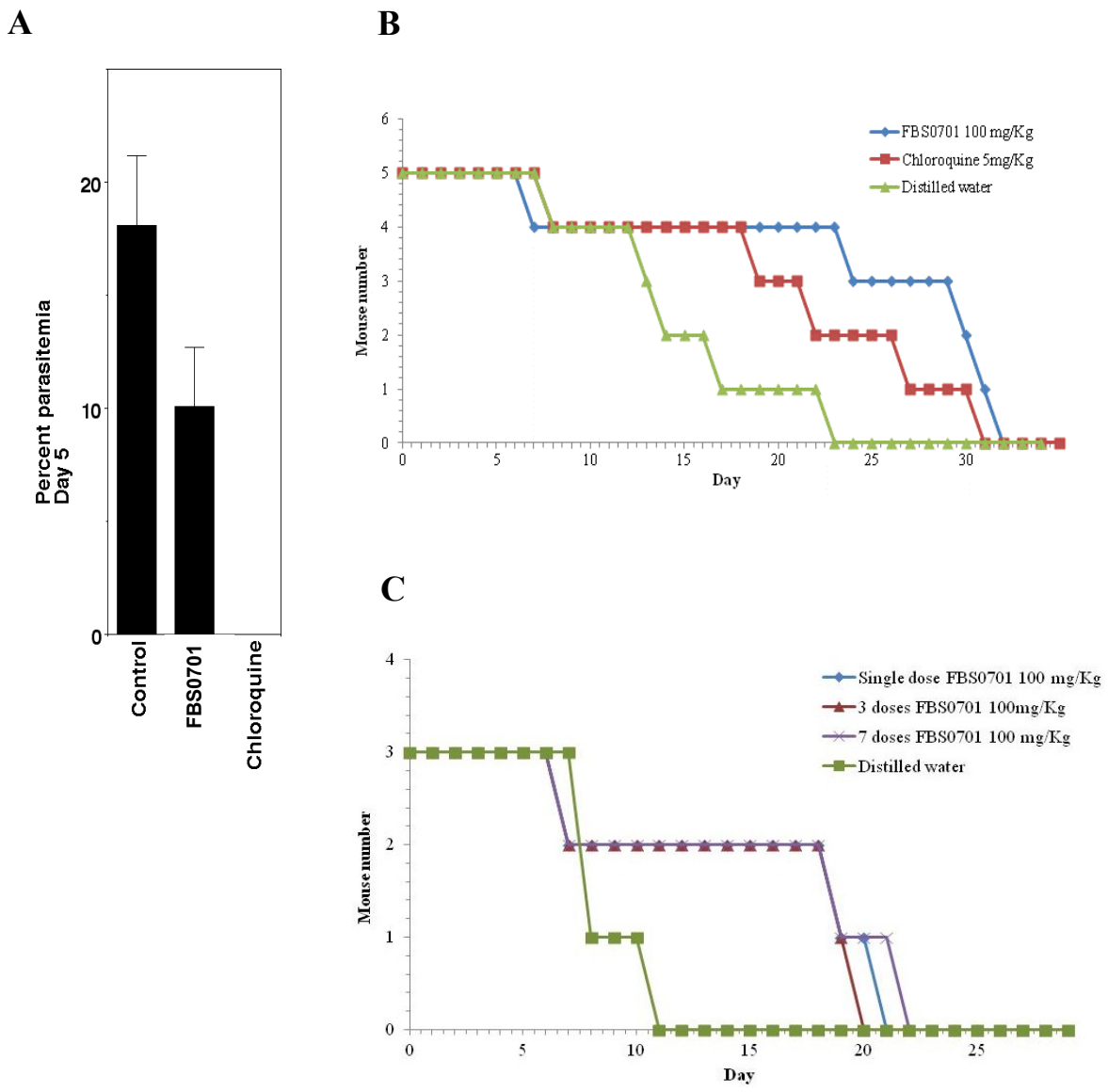


Figure 2.3. FBS0701 delays mouse death after infection with *P. berghei*. We performed a Thompson suppression test in the *P. berghei* mouse model. Mice in groups of five were inoculated with 10 million parasites intraperitoneally and were dose daily with 100 mg/ kg FBS0701 (200 µl) by oral gavage for 4 consecutive days. Parasitemia was determined 5 days after infection by examining 20 fields of blood smears for each of the 5 mice. FBS0701 induced 50% percent reduction in parasitemia and prolonged survival significantly. Five mg/Kg chloroquine dosed daily for 4 days was used as positive control for parasite death (**A and B**). Similarly to test the duration of dosing needed to suppress parasites, 100 mg/Kg FBS0701 was given orally daily for 3 days, 7 days and on a single oral dose. The survival curves were similar for all the three dosing schedules (**C**).

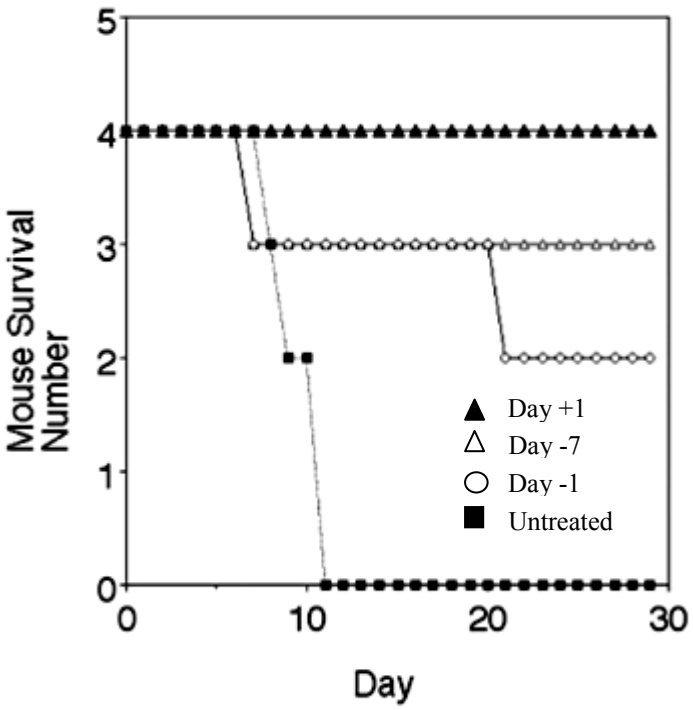


Figure 2.4. FBS0701 cures lethal *P. yoelii* in a single dose. Male mice in groups of 4 were inoculated with 10 million lethal *P. yoelii* parasites by IP injection on day 0. A single oral dose of FBS0701 at 100 mg/kg was given on day -7, day -1 and day +1 relative to infection. The survival curve shows complete protection with day +1 dosing and significant protection in animals pre-treated day -7 and day -1. Treated day +1 (filled triangle), pre-treated day -7 (empty triangle), pre-treated day -1 (empty circle), untreated (filled square).

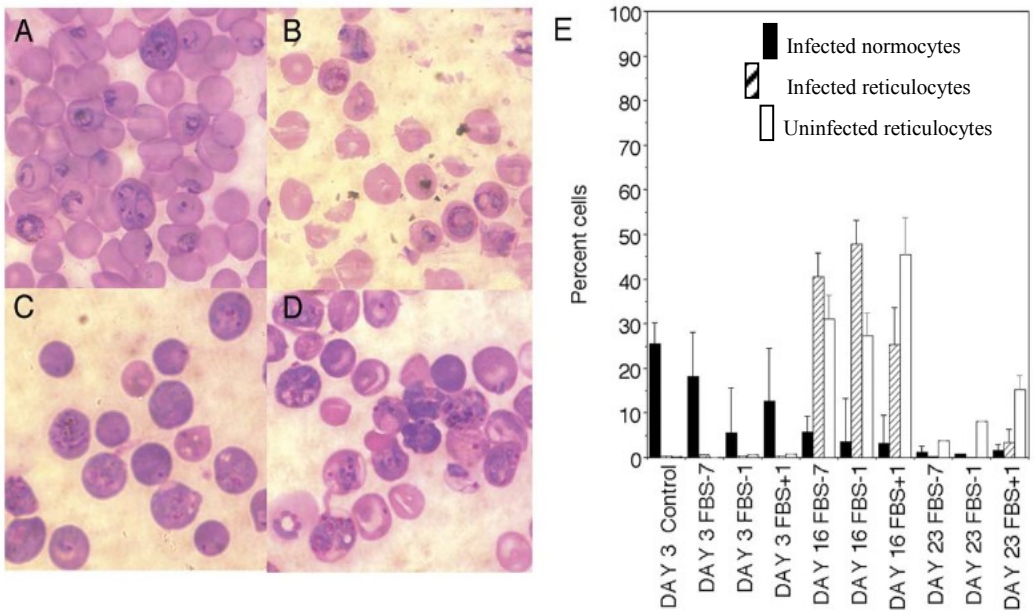


Figure 2.5. Protection is associated with exclusion of infection from normocytes in *P. yoelii* infected mice. On day 3 blood films from control (**A**) and FBS0701 (**B**) treated male mice both show infection in normocytes. On day 16 *P. yoelii* infection is largely restricted to young reticulocytes with only a few in normocytes in mice treated on day -7 (**C**) or day -1 (**D**). Quantification of Giemsa-stained blood films (**E**) indicated that on day 3 infected normocytes (black bars) outnumbered by more than 20 fold infected reticulocytes (hatched bar). On day 16 most of the erythrocytes are either infected (hatched bars) or uninfected reticulocytes (clear bars). By day 23 more than 80 to 90% of the erythrocytes are uninfected normocytes. Parasitemia on day 3 is reduced compared to control animals which are absent in subsequent days due to control animal death. Quantification was performed on counting at least 500 erythrocytes per mouse and averaged in the groups of 4 or number of remaining mice. Counts are represented as number of infected or uninfected normocytes or reticulocytes out of 100. Error is standard deviation of means between up to 4 mice.

2.4.3 Mouse pharmacokinetics

We performed a pharmacokinetic analysis of FBS0701 dosing in fed mice without malaria. One hundred mg/kg dosing resulted in 17 μM levels at one hour that by 8 hours was close to 1 μM (Figure 2.6). These concentrations are below those easily achieved in humans[110].

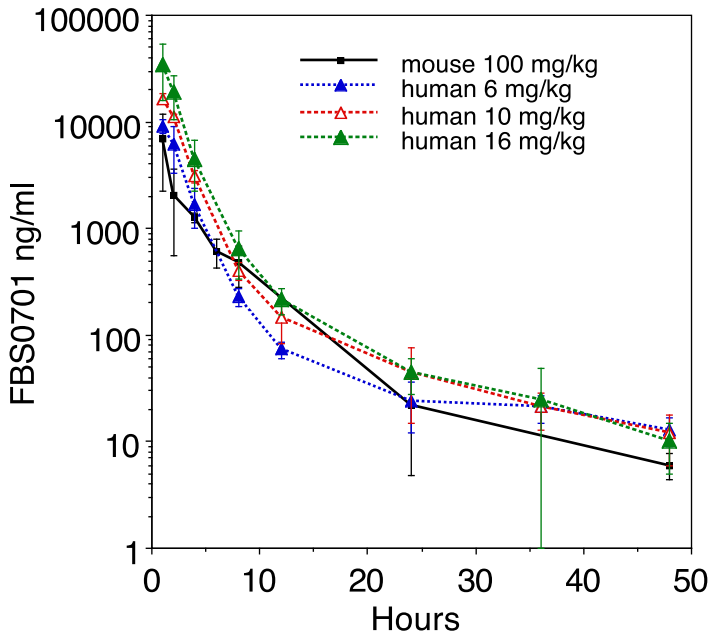


Figure 2.6. Pharmacokinetics of FBS0701 in mice show high peak values but below concentrations for humans. 21 fed mice were given single oral dose at 100 mg/kg (black squares) and were sacrificed at indicated time with whole blood separated into erythrocytes and plasma. At least 200 µl of plasma was analyzed for drug levels in the three mice at each time point. Error is standard deviation of the mean. The published data for FBS0701 kinetics in humans at doses of 6 mg/kg (blue triangle), 10 mg/kg (red triangle) or 16 mg/kg (green triangle) were compared.

2.4.4 Removal of labile erythrocyte iron

Calcein has been used as an intracellular iron probe[137,138]. Labile iron bound to low molecular weight, low affinity chelators binds and quenches calcein to generate the baseline equilibrium fluorescent intensity. The labile iron pools in normal erythrocytes has been determined to be approximately 1 μM[133,30]. Similar to deferiprone, the iron chelator FBS0701 at 100 μM was able to enter erythrocytes as evidenced by the increase in fluorescent intensity consistent with the release of iron from calcein (Figure 2.7A and B). Incubation of the FBS0701 and deferiprone for either one or two hours showed no difference in endpoint fluorescence intensity. To measure the removal of intracellular iron by chelation, erythrocytes at 4 x 10^6 cells/ml (0.125% hematocrit) were incubated for an hour with 100 μM of either deferiprone or FBS0701, washed and then placed in PBS overnight. Figure 2.7C shows that fluorescence in erythrocytes pre-treated with FBS0701 increased slightly from a mean fluorescence intensity (MFI) of 17 to 20. Pretreatment with deferiprone showed a slight though statistically insignificant decrease in MFI.

Addition of either iron chelators to cells previously incubated with FBS0701 showed a small rise of approximately 5 MFI compared to an increase of approximately 15 seen with either PBS or deferiprone-incubated cells demonstrating that in erythrocytes, FBS0701 depletes the labile pool of intracellular iron.

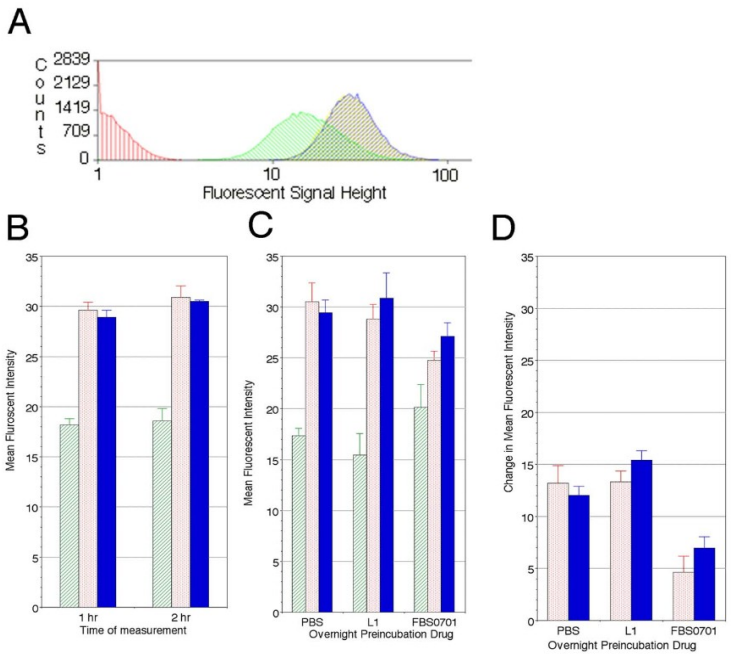


Figure 2.7. FBS0701 treatment chelates intracellular erythrocytic iron to the same extent as deferiprone but unlike deferiprone, removes iron from erythrocytes. Aliquots 1–5 x10^6 erythrocytes from two separate healthy donors were stained in the dark with 0.125 µM calcein-AM for 15 minutes at 37ºC. Cells were washed twice with PBS and allowed to rest for 10 minutes at 37ºC in the dark. Flow cytometry on FACS-Calibur records fluorescent signal height of calcein on 40,000 cells. **A.** Calcein-AM fluorescence histograms of unstained (no calcein) erythrocytes (red) and calcein-stained erythrocytes followed by incubation without (green) or with iron chelator L1 (blue) or FBS0701(yellow) show an increase in fluorescent signal intensity with iron chelator indicating displacement of labile iron from calcein. The L1 and FBS0701 peaks overlap. **B.** Washed erythrocytes after calcein-AM staining were incubated with PBS (green hatched bars), 100 µM L1 (deferiprone) (red dotted bars) or 100 µM FBS0701 (blue solid bars) chelator for 1 hr or 2 hr. Both chelators were able to enter erythrocyte and chelate iron once bound to calcein to increase MFI. **C.** Erythrocytes were incubated with PBS, 100 µM L1 (deferiprone) or 100 µM FBS7010 chelator for 1 hr and washed twice with PBS and incubated overnight in PBS. Erythrocytes were then washed, stained with calcein-AM and incubated with either L1 (red dotted bars) or FBS0701 (blue solid bars). The baseline calcein fluorescence in FBS0701 pre-treated cells was higher indicating a lower concentration of labile iron in these erythrocytes, while baseline MFI in cells incubated with deferiprone have is essentially unchanged. **D.** Change in Mean Fluorescent Intensity is shown for each of the conditions described in **C**. In the FBS0701 pre-treated cells, the addition of 100 µM of either chelator resulted in a change in MFI of 5 compared to a change of 15 in cells treated with either PBS or deferiprone. The data in **B** was performed in three replicates on RBCs from a single blood donor while **C** and **D** was performed using RBCs from two different donors each in triplicate and mean and standard deviation (SD) of the 6 data points is shown. Student's t-test shows a statistical significant difference in the change in MFI for both iron chelators and PBS in **B** and **C**. In **C**, baseline FBS0701 MFI is higher than both PBS and deferiprone ($p<0.05$) and in **D** the change in MFI with FBS0701 after the overnight pre-treatment is less than with PBS and deferiprone (student's t-test, $p<0.005$).

2.5 Discussion

A bioavailable oral iron chelator is an attractive and proven objective as an antimalarial agent. Much of the lack of efficacy of previous iron chelators for malaria therapy can be attributed to pharmacokinetic limitations including bioavailability, poor iron clearance efficiency and short-half life. FBS0701 has high solubility and a *log p* of -1.22 permitting good oral bioavailability and much better iron clearance than deferoxamine and deferiprone. Here we demonstrate activity against blood stage *P. falciparum* and also single dose cure in a lethal mouse malaria model. A potential limitation is the known interference to the artemisinin class of drugs. However, the evidence from this work indicates that FBS0701 may remove an irreplaceable source of iron in normocytes in mice. Previous studies on where iron is removed with deferoxamine in the calcein measurements showed a decrease in labile iron in both infected and uninfected erythrocytes. We have not demonstrated a decrease in nonheme iron in these studies but suggest that the effect we see *in vivo* is not from a new recompartmentalization of iron but from a decrease in erythrocyte levels. Consistent with other reports[126,131,135], we have demonstrated *in vitro* interference when both iron chelators and artemisinin are present simultaneously, but have not tested whether pre-treatment with FBS0701 before infection interferes with artemisinin activity. Interestingly, the dose of iron chelator largely restricted lethal *P. yoelii* to young erythrocytes even almost 28 days after the drug was dosed and three weeks into infection. Mouse erythrocytes have a life span of about 60 days[139,140]. Additional studies of the timing of iron chelator days to weeks before lethal *P. yoelii* infection will provide evidence to replenishment of this erythrocyte bioavailable source of iron for *Plasmodium*.

The mouse pharmacokinetic parameters are close to those obtained in the rat model[128]. Most of the iron-FBS0701 complex was excreted in the bile while free FBS0701 has renal excretion[128]. The relative level of FBS0701 in the rodent shows liver> kidney> plasma> pancreas> heart.

Interestingly, humans show approximately ten times higher plasma concentrations compared to mouse for a given mg/kg dose. However, in studies comparing dose proportionality for cancer drugs where toxicity is a larger issue 100 mg/kg dose in mice represents a dose of 300 mg/m^2 while a 10 mg/kg in humans is equivalent to 370 mg/m^2 [141]. The implications are that in humans, effective drug concentrations above minimal *P. falciparum* inhibition concentrations last less than ten hours. In the *P. falciparum in vitro* drug testing the drug is at continuous concentration for three days. In the mouse *in vivo* studies here we have demonstrated a persistent effect weeks after the drug was dosed.

This data indicate that iron chelation is able to remove a bioavailable source which persists and plays a role in parasite inhibition. Unlike many of the long half-life blood stage active quinolines which rely on time above inhibition concentration to kill parasites, FBS0701 has an effect possibly by perturbing an iron compartment in the circulating erythrocytes. The determination of relative labile iron concentrations using calcein indicates FBS0701 is able to enter erythrocytes and bind iron bound to calcein.

More importantly, in contrast to deferiprone, FBS0701 was able to remove labile iron from erythrocytes, an effect that persisted for at least 16 hours. This is the first study to demonstrate the egress of labile iron complexed with a chelator complex from erythrocytes. This has important clinical implications for the use of an iron chelator in the

treatment of malaria as well as the ability of FBS0701 to remove iron from other cell types.

In the malaria iron chelator literature there is some debate whether iron chelators inhibit by limiting DNA replication which in many bacteria and mammalian cells is cytostatic or whether iron chelators are toxic and therefore parasiticidal to *Plasmodium*.

Early stage gametocytes do not replicate DNA, so the data which show early stage inhibition with FBS0701 may indicate an additional toxic mechanism of inhibition at the high dose used in the gametocyte assay at the stages digesting the iron and hemoglobin rich erythrocyte cytoplasm. In summary, FBS0701 demonstrates more potent inhibition for *P. falciparum* than previous iron chelators in clinical use. The activity persists in mice after the drug is below inhibition concentrations of approximately 5 μM. Our gametocyte data suggest a combined toxic mechanism in presence of erythrocyte cytosol ingestion in addition to the proposed mechanism of limiting DNA synthesis. In the lethal *P. yoelii* murine malaria model we show single oral dose cure. The removal of erythrocytic iron from intracellular bioavailable pools likely contributes to both the antimalarial activity and duration of effect of FBS0701 accounting for these effects in the relative absence of drug in plasma. FBS0701 may find clinically utility as monotherapy, a malarial prophylactic or, more likely, in combination with other antimalarials after further preclinical testing to investigate liver stage activity, duration of blood stage interference with the artemisinins or possibly inhibition of transmission in mosquito stages ingesting FBS0701 exposed gametocytes.

Chapter 3

IRON AND LIVER STAGE MALARIA

3.1 ABSTRACT

Iron is necessary for *Plasmodium* development, during blood and hepatic stages. The coincidence of malaria and iron deficiency makes it more difficult to treat both conditions as iron supplementation can increase malaria disease and malaria disease decreases iron absorption. In fact, some studies and programs on iron supplementation had to be stopped because of an increase in malaria mortality rates.

Here we confirmed the importance of iron by evaluating the effect of the new iron chelator FBS0701 on the murine malaria hepatic stage. FBS0701 reduced the parasite load and could also act in a synergistic manner with another antimalarial, primaquine. Also, we studied the differences on hepatic infection loads on transgenic anemic mice: overexpressing hepcidin with reduced iron stores in the liver and hemoglobin deficient mice with normal hepatic iron stores. We found lower parasite numbers on mice chronically overexpressing hepcidin. The anemic mice with reported normal liver iron had no changes in liver parasite number.

Finally, we studied the effect of low and high iron diets on the hepatic stage of murine malaria and the interplay of hepcidin with the hepatic infection levels. We used mice that were both anemic and iron deficient by ZnPPIX levels as well as non-anemic, iron replete mice and gave both groups low and high iron diets for 2 and 6 weeks. Our results showed that high iron diets increased liver stage parasites in non-anemic mice and that iron supplementation predominated over a negative hepcidin effect. With our research we partially replicated in mice the human findings from the Pemba study. We began to see the increase in liver stage parasite numbers after 2 weeks which became

more significant after 6 weeks of the high iron diet. While the liver stages are asymptomatic, increasing the number of *Plasmodium* liver stages may synergize with blood stage increases after iron supplementation to increase malaria morbidity and mortality.

3.2 INTRODUCTION

Each year approximately 500 million cases of human malaria occur, with most morbidity and mortality in children under five in SubSaharan Africa[142]. Malaria infection coincides geographically with iron deficiency anemia. Malaria contributes to anemia by decreasing intestinal iron absorption, restricting erythropoiesis and sequestering bioavailable iron in ferritin stores[6]. Prevalence of anemia can be as high as 95% in malaria highly endemic areas like Pemba[143]. Studies examining malaria and anemia have resulted in contradictory findings. Some older studies associated injectable iron or oral refeeding in famine stricken refugees with a high percentage of symptomatic malaria[118,119]. However, other studies including a meta-analysis did not show exacerbation of human malaria with oral iron replacement[120,144,143,145,146]. Policy on iron supplementation of anemic individuals has been intensely debated after the study in the Island of Pemba, hyperendemic for malaria. Results of this study showed that both iron and folate supplementation together increased the risk for hospitalization and death presumed to be from malaria in iron replete rather than iron deficient children regardless of anemia[112]. The supplementation had a protective effect in children who were both iron deficient (elevated ZnPPIX as a metric) and anemic; no effect in children iron deficient

and with no anemia, but an apparent significant risk of adverse outcomes and deaths in children iron replete with or without anemia.

Iron is important for most living organisms including *Plasmodium* species. Iron supplementation increases hepatic parasite number and iron chelators decrease the development of these exoerythrocytic forms [9]. The level of hepcidin, an important iron regulator, is increased in blood stage malaria. Hepcidin limits liver stage infection levels[9] and induces ferroportin degradation. Without ferroportin, iron absorption is limited from intestinal cells and so does its release from recycling macrophages during malaria [34,147,148]. Hepcidin is also induced by inflammation [56,149] and down-regulated by hypoxia or iron deficiency[150]. The biologic mechanisms and timing affecting iron supplementation as well as the interplay of hepcidin and iron supplementation over time has not been defined in the context of iron-deficiency anemia and liver stage malaria.

In the present study, we tested the effect of the new iron chelator FBS0701 on the liver stage as well as two different genotypic mice with predicted low and normal iron stores. We also used anemic iron-deficient and non-anemic iron-replete mice to compare the effect of high, normal and low iron diets and timing of the iron supplementation on hepatic stage malaria parasite numbers.

3.3 Methods

3.3.1 Materials

Special mouse chow was ordered from (Harlan, Madison, WI). Low iron diet (TD.99397) had 2-6 mg iron/kg, normal iron diet (TD.08713) had 200 mg iron/kg and high iron diet (TD.08714) had 20 g iron/kg. FBS0701 produced under good manufacturing practice was obtained from Aptuit (Kansas City) LLC.

3.3.2 Animal care

Five month old female Balb/c mice were purchased from Jackson Laboratories (Maine, USA) and maintained in the Facility of Johns Hopkins Bloomberg School of Public Health. Mice were maintained in a low iron diet for five months. Food and distilled water was provided ad libitum. Because mice did not develop iron deficiency on the low iron diet, phlebotomy was performed with removal of 500 µl of blood three times. Three weeks after phlebotomy, mice were confirmed to be anemic by measuring Hb and ZnPPIX/heme ratio. Two days post anemia confirmation; groups were divided according to the different iron diets. A control non-anemic and an anemic group were included for each type of diet. Six groups were maintained on the new diets for 2 weeks and other 6 groups for 6 weeks. Anemia status was checked on day of liver harvest for the two-week diet experiment mice whereas the status on those with 6 weeks of diet were tracked 2 times before liver harvest and on day of sacrifice.

Female hepcidin Tg+ mice (C57BL/6 background) and female hemoglobin deficient or hbd mice (C57BL/6 background) were provided by Dr. Cindy Roy (JHMI). Hepcidin Tg+ mice carry both a tTA transgene (tetracycline transactivator protein) and a TRE.*Hepc1* transgene (tetracycline response element fused with the hepcidin gene). The TRE.*Hepc1* transgene is "off" in the presence of tetracycline. We maintained the gene "on" throughout the study without the addition of tetracycline. The hepcidin transgene is over expressed while endogenous hepcidin is still produced. These mice mirror characteristics of anemia of chronic disease with a mild microcytic hypochromic anemia and mild elevation of reticulocytes counts. Their erythrocytes have a normal life span. Tg+ mice have decreased iron in hepatocytes with a shift of iron to the spleen and sequestration of iron in macrophages[151].

Hbd mice have an exon deletion in gene Sec15l1 (protein involved in the cycling of transferrin) making the uptake of transferrin iron defectuous in the erythroid precursor. Sec15l1 is homologous to a gene encoding a member of the exocyst pathway in yeast. These mice are hypochromic, with a decrease in hemoglobin and normal liver iron stores[152]. Female C57BL/6 mice were used as control. Human exogenous hepcidin-25 trifluoroacetate salt (Bachem, Bubendorf, Switzerland) at 1mg/ml was given intraperitoneally to a group Tg+ mice and C57BL/6 mice 4 hours before *P. berghei* inoculation.

Male C57BL/6 mice were used for testing FBS0701 and were maintained in the Facility of Malaria Unit of University of Lisbon.

3.3.3 Animal ethics

All experiments were performed according to protocol "Iron and Murine Malaria" (Approval ID-MO09H403) approved by The Johns Hopkins Animal Care and Use Committee in accordance with institutional standards.

3.3.4 Induction of anemia by phlebotomy

Whole blood was collected by submandibular/facial bleeding. A Medipoint golden mouse lancet (Medipoint, Mineola, NY) sized 4 was used to stick mice in the temporal vein which traverses above the check pouch. The lancet allowed us to stick with enough pressure to insure a good blood draw making puncture as deep as the point of the lancet. Mice were bled in total 3 times with intervals of 3 days and then allowed to recover during 3 weeks. Every time ~500 µl of blood was collected with a subsequent replacement of the same volume with PBS (1X) by intraperitoneal route. Blood was stored at -80°C.

3.3.5 Real-time *in vivo* imaging of luminescent *Plasmodium* on FBS0701 treated mice

Thirty-six hours post infection with luciferase-expressing *P. berghei* parasites (parasite line 354cl4), shaved abdominal C57BL/6 mice were injected with 200 µl D-luciferin dissolved in PBS (150mg/Kg; Caliper Life Sciences, USA) subcutaneously in

the neck. After five minutes, mice were injected intraperitoneally with 150 µl of Avertin to be anesthetized. Five minutes later, biolumiscence imaging was detected with a 12.5 cm field of view (FOV), a medium binning factor and 180 seconds of exposure. For luciferase activity, mice whole bodies were visualized with the IVIS Imaging System 100 (Xenogen- Caliper Life Sciences, USA).

3.3.6 qReal-Time PCR quantification of liver infection and murine hepcidin level

Balb/c, Hepcidin Tg+ and hbd mouse livers were collected and homogenized in TRizol (Invitrogen, Carlsbad, CA) and stored at -80C. Total RNA was isolated by adding chloroform and isopropanol and resuspending the RNA pellet in 75% ethanol. Pellets were finally dissolved in DEPC treated water and stored at -80C. Reverse transcription was performed with: random hexamers (50 µM), PCR buffer (1X), MgCl2 (5mM) dNTPs (4mM), RNAse inhibitor (1U/µl) and MuLV reverse transcriptase (2.5U/µl) from Applied Biosystems, Branchburg, NJ. The conditions for cDNA synthesis were: 25°C for 10 min, 42°C for 20 min and 95°C for 5 min.

Liver infection and murine hepcidin level were quantified by using *P. berghei* ANKA (PbA) 18s rRNA and Mouse *hamp* specific primers respectively. Multiplex qRT-PCR reactions included 1X IQ Multiplex Power Mix (BioRad, Hercules, CA), 0.2 µM 18s and hamp primers, 0.5 µM Hypoxanthine Guanine Phosphoriboayltransferase (hprt) primers (Gagliardi et al., 2011), 0.2 µM of each TaqMan probe and 3 µl (around 540 ng/µl) cDNA for a total volume of 10 µl. Cycling conditions were followed according to

the BioRad CFX96: 95C for 3 min and 39 cycles of 95C for 10 sec and 55C for 30 sec. The gene copy number was normalized against the hprt housekeeping gene. The specific sequences of primers (Sigma Aldrich, Saint Louis, MO) were: 5'-GGAGATTGGTTTTGACGTTTATGCG-3' and 5'-AAGCATTAAATAAAGCGAATACATCCTTA-3' for 18s; 5'-TGCAGAAGAGAAGGAAGAGAGACA-3' and 5'-CACACTGGGAATTGTTACAGCATT-3' for *hamp* and 5'-TCCCAGCGTCGTGATTAGC-3' and 5'-CGGCATAATGATTAGGTATACAAAACA-3' for hprt. The specific sequences for the probes (Integrated DNA Technologies, Coralville, IO) were: 5' 6-FAM/ZEN-CAATTGGTTTACCTTTTGCTCTTT-3'IBFC for 18s; 5' TET-CAACTTCCCCATCTGCATCTTCTGCTGT- 3'IBFQ for *hamp* and 5' Cy5-TGATGAACCAGGTTATGACC-3'BHQ-2 for hprt. Standardization curves were made with cDNA plasmids cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA).

C57BL/6 mice livers were collected and homogenized with denaturing solution (4M guanidine thiocyanate, 25mM sodium citrate pH 7, 0.5% N-lauroylsarcosine and 0.7% beta-mercaptoethanol and DEPC water) and stored at -80C. Total RNA was isolated by using the RNeasy Mini kit (Qiagen, MD). Reverse transcription was performed with the Transcriptor First Strand cDNA synthesis kit (Roche, Mannheim, Germany): random hexamers (60 μM), Transcription RT reaction buffer 1X, dNTPs (4mM), Protector RNAse inhibitor (20U) and Transcriptor Reverse Transcriptase (10U). The conditions for cDNA synthesis were: 25°C for 10 min, 55°C for 30 min and 85°C for 5 min.

Liver infection was quantified by using PbA 18s rRNA specific primers. qRT-PCR reactions included: 1X Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) 0.2 µM 18s primers and 2 µl cDNA for a total volume of 20 µl. For normalization 18s primers were replaced by 0.2 µM Hypoxanthine Guanine Phosphoriboayltransferase (hprt) housekeeping gene primers in the reactions. Cycling conditions were as follow: 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min.

The specific sequences of primers were: 5'-CGG CTT AAT TTG ACT CAA CAC G-3' and 5'-TTA GCA TGC CAG AGT CTC GTT C-3' for PbA 18s rRNA and 5' – TGC TCGAGA TGT GAT GAA GG – 3′ and 5' – TCC CCT GTT GAC TGG TCA TT – 3' for mouse hprt.

3.3.7 Sporozoite infection

Female mice were infected with 15,000 *P. berghei* ANKA sporozoites by tail vein injection. Thirty six hours post infection; mice were sacrificed with inhalation overdose of isoflurane. Livers were collected for qRT-PCR quantification of *P. berghei* infection load and mouse hepcidin expression and heart blood was collected for determination of anemic status.

C57BL/6 male mice were infected with 10,000 luciferase - *P. berghei* sporozoites by eye injection post anesthesia with isoflurane. Thirty six hours post infection and post

bioluminescence assay infected mice were sacrificed with inhalation overdose of isoflurane. Livers were collected for qRT-PCR quantification of parasite infection load.

3.3.8 FBS0701 testing

Freshly made solutions of FBS0701 in water were administered by oral cannulation to C57BL/6 male mice. The effective dose of the drug was tested at 4 different concentrations: 50, 100, 200 and 400 mg/kg. The interaction between FBS0701 and primaquine was tested by reducing to half the effective dose of primaquine (from 30mg/Kg to 15mg/Kg) and FBS0701 at 400 mg/kg. The interaction of the drugs was also evaluated at the level of survival. Blood was taken from the tail for smears and parasitemias were followed for 24 days. Parasitemias were determined in a blinded fashion by counting 4 fields of approximately 200 erythrocytes per field. The effective schedule of drug administration was tested at 400 mg/Kg as follows: -1; 0; +1; -1, 0, +1; -1, 0; 0, +1 days relative to infection on female Balb/c mice.

3.3.9 Iron assays

Blood was collected by retro-orbital bleeding from Avertin anesthetized mice into EDTA microtainer tubes (BD, Franklin Lakes, NJ). Mouse hemoglobin levels were determined based on the absorbance of samples at 540 nm using the Drabkin's reagent (Sigma, St Louis, MO) following the instructions provided by the manufacturer. An aliquot of blood was placed on a cover glass (Aviv Biomedical Inc., Lakewood, NJ) to

quantify the ZnPPIX/heme ratio on the ZPP hematofluorometer (Aviv Biomedical Inc., Lakewood, NJ). Normal mouse ratios of ZnPPIX/heme in the literature range from 80 to 120 μM ZnPPI / M heme [153,154,155]. Livers and spleens from *P. berghei* infected female Balb/c mice were collected 2 days post infection and stored at -80C. Tissue nonheme iron was quantified as described by Cook with minor modifications[156]. Briefly, tissues were accurately weighted and digested on an acid solution containing HCl 3 M and 0.61M trichloroacetic acid for 50 h at 65ºC. Fifty microliters of the tissue extract were added to 1 ml of working chromogen solution (5 vol of saturated sodium acetate, 5 vol of iron free water and 1 vol of chromogen stock containing 1.86 mM bathophenanthroline sulfonate and 143 mM thioglycolic acid). Samples were incubated for 10 minutes at room temperature. Absorbance was determined at 535 nm. An iron standard curve diluted on acid solution was made with ranging concentrations from 200 μg/dl to 1000 μg/dl. Measurements were expressed as micrograms of iron per gram of tissue.

3.3.10 Statistical analysis

To observe the effect of hepcidin on parasite level we displayed the means of parasite across four groups of mice: (a) normal mice, (b) normal mice that received hepcidin, (c) overexpressing-hepcidin-transgenic mice, and (d) overexpressing hepcidin transgenic mice that received hepcidin.

We performed simple linear regressions to model the relationship between diet and parasite level, and between diet and hepcidin level at 2 weeks and at 6 weeks after

receiving the iron diet. In all cases we stratified the regressions by anemic status to compare the slopes and to detect the effect modification caused by anemia. To regress hepcidin on iron in diet, we used a spline with a knot at iron = 200 µg, which corresponds to the normal diet group. We transformed values of parasite level, hepcidin level, and iron in diet to log10 scale to control for the right skewness of the data. All analyses and graphs were produced in R* and all statistics were evaluated at an alpha level of 0.05[157].To analyze the experiments of FBS0701 effect on liver infection and transgenic mice we used a t-Student test and a Wilcoxon test. Statistics were evaluated at an alpha level of 0.05.

3.4 Results

3.4.1 FBS0701 reduces *Plasmodium* liver parasite numbers

To study of the importance of iron for the hepatic stage of the parasite we tested the effect of the iron chelator FBS0701 which has a hepatic clearance. We found a dose-response on decreasing parasite load as shown by the bioimaging assay (Figure 3.1) and a statistically significant reduction between the non treated (0 mg/kg) and the FBS0701 treated mice at 400 mg/kg (t-Student test=3.25; p-value=0.043).

To test the effect of complete clearance of parasites, mice received FBS0701 at 1000 mg/kg and showed a statistically significant reduction of liver infection. Parasite load was evaluated by bioimaging (Figures 3.2A-B, t-Student test = -4.6619; degrees of

freedom = 5.77; p-value = 0.004) and by qReal-time PCR to make it more accurately (Figure 3.2C, t-Student test = -3.42; degrees of freedom = 4.18; p-value = 0.025).

Results showed that mice treated with FBS0701 and primaquine combined induce a significant reduction (Wilcoxon test statistic = 16; p-value = 0.02857) of the parasite load compared to control. Primaquine or FBS0701 alone induced a reduction of parasite load. However, the difference between primaquine or FBS0701 and the untreated control was not statistically significant, likely because of the small sample size (Figure 3.3). The positive effect of the combination therapy was also reflected on a more prolonged survival. Mice from the non treated (control), FBS0701 and primaquine groups alone showed symptoms of cerebral malaria. Mice began dying on these groups from day 6 post infection whereas those mice receiving combined drugs survived for 23 days post-infection, although they were sacrificed for being very weak and anemic at that time (Figure 3.4).

Also, FBS0701 was found to be more effective given at one day post liver stage infection, similar to the treatment of murine blood stage infection[158](Figure 3.5).

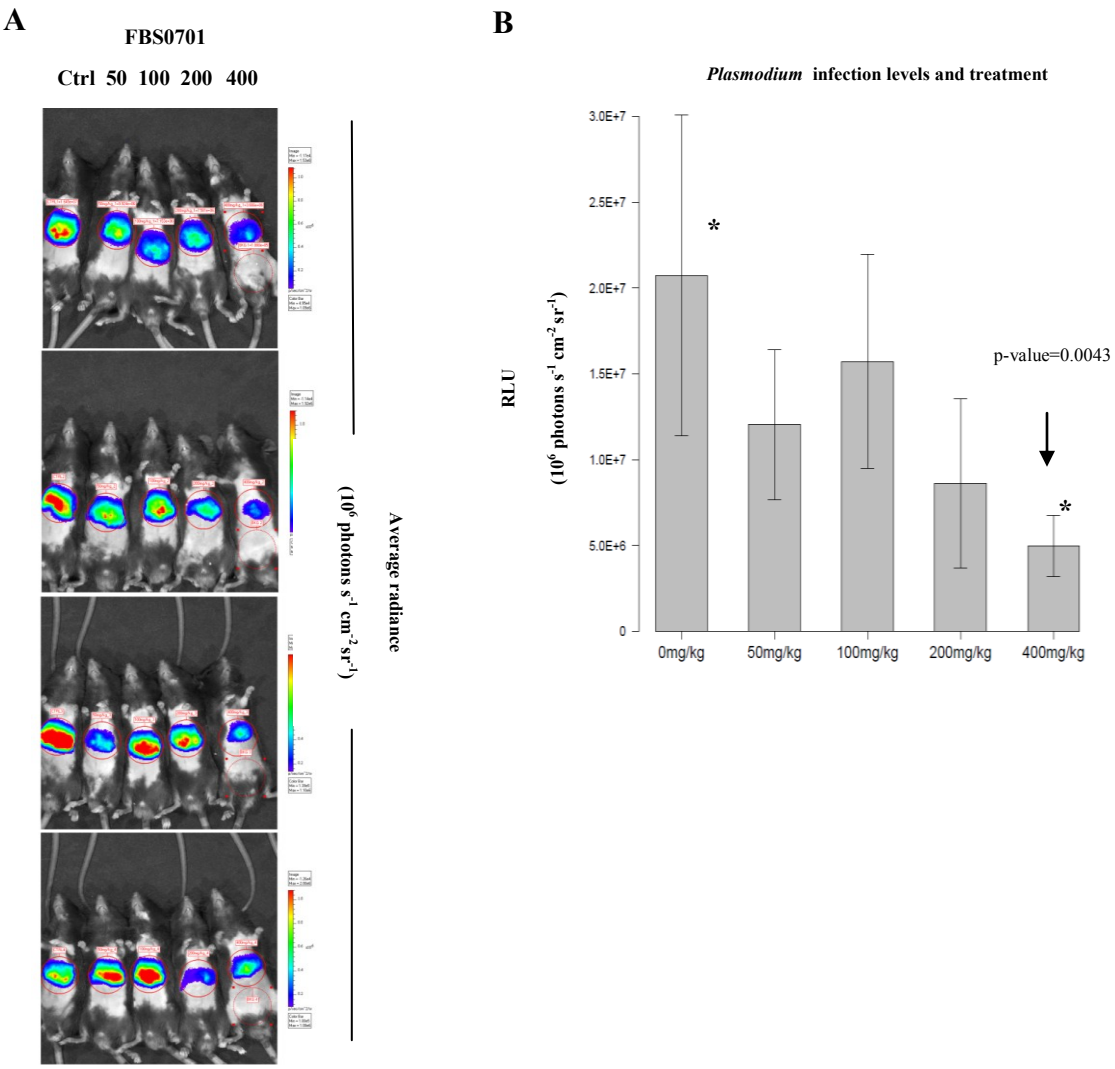


Figure 3.1. FBS0701 showed a dose-response behavior on infected mice. Male C57BL/6 mice in groups of 4 were treated with FBS0701 at concentrations of 50, 100, 200 and 400 mg/kg. Control mice (0mg/kg) did not receive any drug at all. Initially mice were infected with 10000 Luciferase – expressing *P. berghei* sporozoites by eye injection. Drug was given orally 4 hours before infection. Forty hours after infection, control and treated mice were injected with luciferin upon anesthesia and their hepatic infection load was quantified by luminescence and expressed by Relative Luminescence Units (RLU)**.** All treatment groups were compared to the control group with t-Student test. The group that received 400 mg/kg FBS0701 reported the lowest level of infection and was the only group that was statistically different than the control group (t-Student test=3.25; p-value=0.043)**.** Asterisks show the groups that were statistically significant **A.** Real time *in vivo* imaging of infected mice by luminescence. Each panel represents all the control (Ctrl, no drug) and treated mice according to 50, 100, 200 and 400 mg/kg doses. **B**. Distribution of luminescence by treatment group.

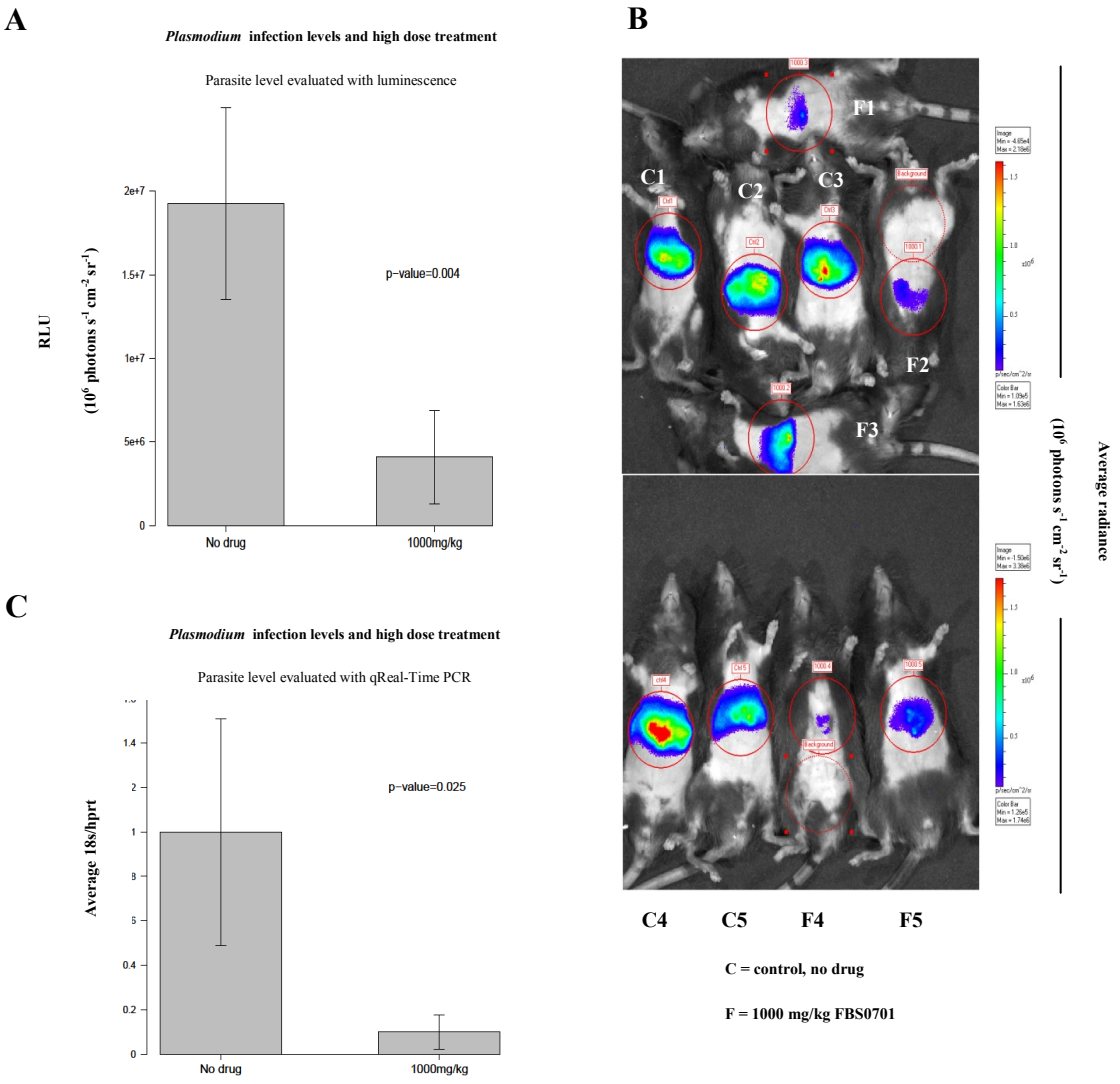


Figure 3.2. FBS0701 at 1000mg/kg induced a significant decrease of hepatic parasite close to clearance. Male C57BL/6 mice in groups of 5 were treated with FBS0701 at 1000 mg/kg (mice F1 –F5) or were left untreated (control mice, C1-C5). Initially mice were infected with 10000 Luciferase – expressing *P. berghei* sporozoites by eye injection. Drug was given orally 4 hours before infection. Forty hours after infection, control and treated mice were injected with luciferin upon anesthesia and their hepatic infection load was quantified by luminescence and expressed by Relative Luminescence Units (RLU) and were later sacrificed to collect their livers for parasite quantification by qReal-Time PCR. **A-B.** Real time *in vivo* imaging by luminescence of infected mice (t-Student test = -4.6619; degrees of freedom = 5.77; p-value = 0.004). Each panel on **A** represents all the control (C, no drug) and treated mice with 1000 mg/kg of FBS0701 (F). **C.** qReal –Time PCR quantification of infected mice (t-Student test = -3.42; degrees of freedom = 4.18; p-value = 0.025).

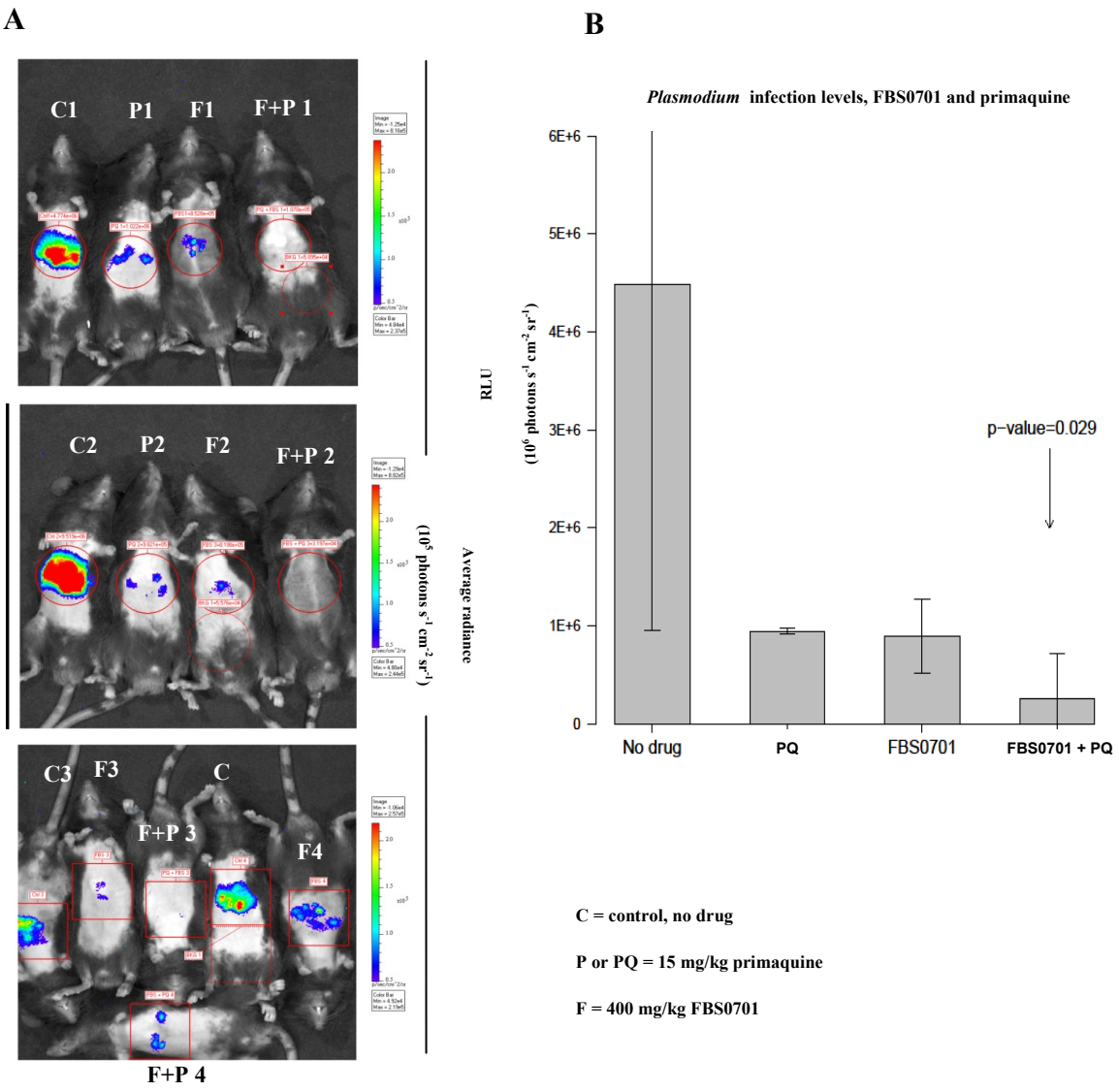


Figure 3.3. FBS0701 and primaquine combined induce a significant hepatic parasite decrease in comparison to their effects when administered alone. Male C57BL/6 mice in groups of 4 were treated with FBS0701 (F) at 400mg/kg and/or primaquine (PQ) at 15mg/kg. Control mice (ctrl) did not receive any drug at all. Mice were infected with 10000 Luciferase – expressing *P. berghei* sporozoites by eye injection. Drugs were given orally 4 hours before infection. Forty hours after infection, control and treated mice were injected with luciferin upon anesthesia and their hepatic infection load was quantified by luminescence and expressed by Relative Luminescence Units (RLU). The effects of FBS0701 and primaquine alone did not differ statistically between them. During the experiment, 2 mice died in the group that received primaquine alone. Each treated groups was compared against the control group (no drug). Asterisks indicate the groups that were statistically significant. **A.** Real time *in vivo* imaging of infected mice by luminescence. Each panel represents all the control (Ctrl, no drug) and treated mice according with primaquine (P) or FBS0701 (F) or combined (P + F) **B.** Analyzed data from A. (Wilcoxon test statistic = 16; p-value = 0.02857).

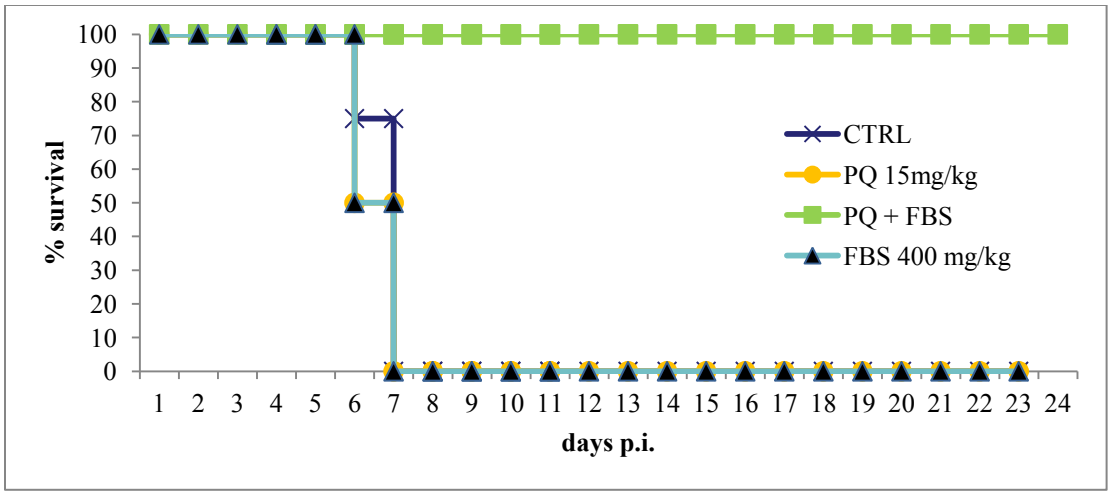


Figure 3.4. Mice that were treated with FBS0701 and Primaquine combined showed a higher survival. Male C57BL/6 mice, from the previous experiment, infected with 10000 Luciferase – expressing *P. berghei* sporozoites were tracked until the last mouse died or had to be sacrificed for its weakness. Mice that received FBS0701 (FBS) and primaquine (PQ) combined survived until day 24, when they had to be sacrificed for being severely sick and anemic from malaria. Mice that received drugs alone or no drug started dying 6 days post infection.

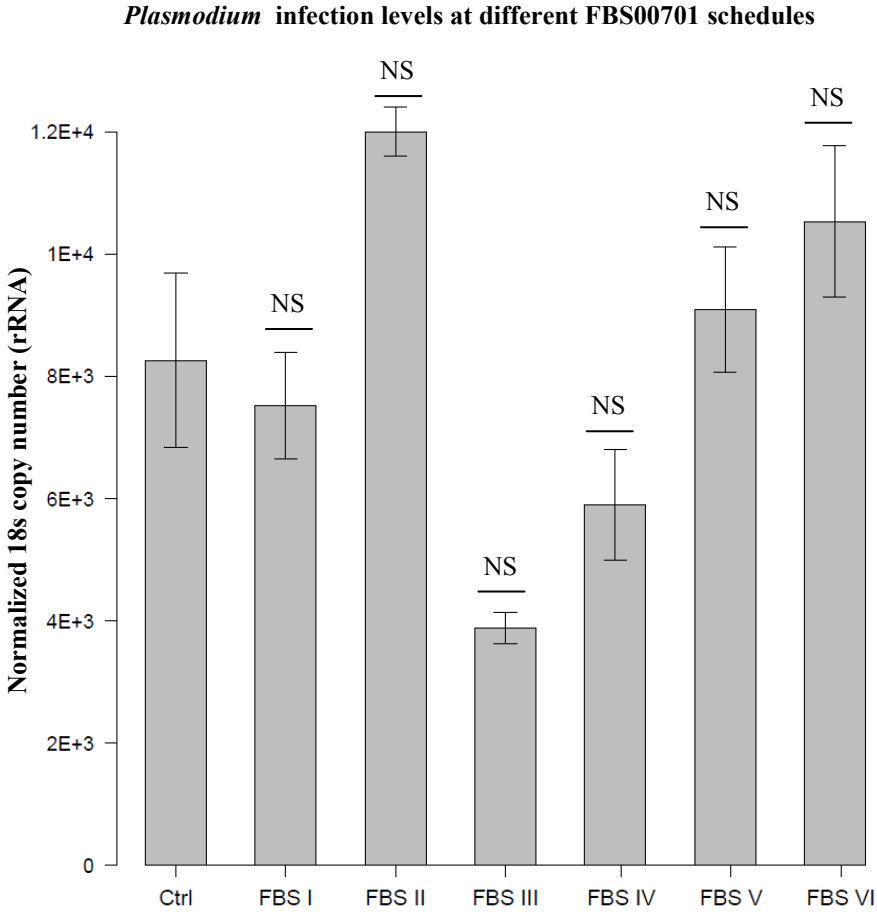
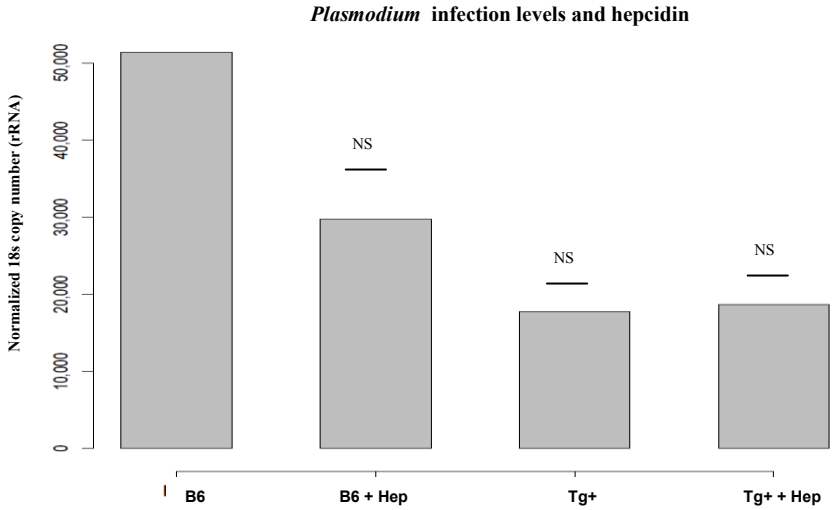


Figure 3.5. FBS0701 showed to be more effective at controlling hepatic parasite load when given one day post infection. Schedule of supplementation of FBS0701 (FBS). Days relative to infection: I, -1 day; II, 0 day; III, +1; IV, -1,0,+1 days; V, -1,0 day; VI, 0,+1. FBS0701 was given at 400 mg/kg orally. Control mice did not receive drug at all. Mice in groups of 5 were infected with 15000 *P. berghei* sporozoites by tail vein injection. Forty hours after infection, control and treated mice were sacrificed to collect their livers for parasite quantification by qReal-Time PCR. Parasite load average and standard deviations are represented. Data was not statistical significant compared to control. NS = not significant.

3.4.2 Transgenic hepcidin mouse (Tg+) and hemoglobin deficient mouse (hbd) malaria indicate that hepcidin controls bioavailable iron important for hepatic parasite development

We next studied a group of transgenic hepcidin mice (Tg+), which has lower liver iron stores, and a hemoglobin deficient murine group that has normal liver stores but is anemic from an erythrocyte defect in the iron/transferrin uptake. The study showed that in comparison to C57BL6 control mice, overexpressing hepcidin transgenic mice (Tg+ mice) resulted in a lower parasite load with a similar effect from receiving extra exogenous hepcidin (Figure 3.6); however, we did not find a statistically significant difference in this analysis (Kruskal-Wallis chi-squared = 4.6287, degrees of freedom = 3, p-value = 0.20). Hemoglobin deficient mice showed a non-significant difference (t-Student test=1.46; p-value = 0.21) between their loads of *Plasmodium* infection compared to C57BL6 control mice. Normalized hepcidin levels in hbd mice were 1,97E+04 (SE 5.86E+03) vs. 2.31 E+04 (SE 1.23E+04) in C57BL6 mice (Figure 3.6).

A



B

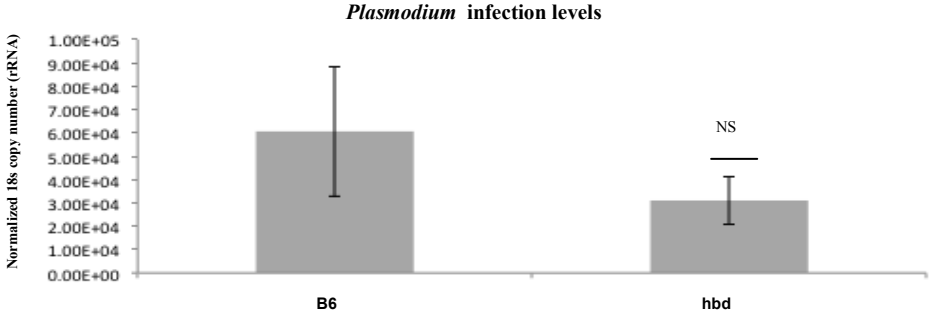


Figure 3.6. Transgenic hepcidin mice after receiving exogenous hepcidin showed a lower parasite load while parasite loads on hemoglobin deficient mice were not different compared to control mice. **A.** Two groups (4 each) of anemic transgenic hepcidin mice (Tg+), and two groups (4 each) of non-anemic wild type mice (C57BL6 or B6) were infected with 15,000 *P. berghei* sporozoites by tail vein injection. On day of infection one wild type group (C57BL6 + hep) and one transgenic group (Tg+ + hep) received exogenous hepcidin (1 mg/ml) by intraperitoneally. Two days after infection mice were sacrificed and theirs livers were collected to quantify hepatic infection level by qReal time PCR. We found a negative trend of parasite load with overexpression of endogenous and exogenous hepcidin and the combination of both (Kruskal-Wallis chi-squared = 4.6287, degrees of freedom = 3, p-value = 0.20). **B.** A group of 4 non-anemic mice wild type mice (C57BL6) and 4 hemoglobin deficient (hbd) mice were infected and analyzed as described on **A**, however none of the mice received exogenous hepcidin. Hbd mice showed a non-significant difference between their parasite loads compared to control mice (t-Student test=1.46; p-value = 0.21). Normalized hepcidin levels in the liver of hbd mice were 1.97E+04 in the setting of severe anemia. Additional information: Hb is 9.9 g/dl and HCT % is 38 on hbd mice; Hb is 12.6 g/dl and HCT% is 42 on C57BL6 mice. NS = not significant

3.4.3 Phlebotomy induces iron deficiency anemia in mice

We obtained just weaned Balb/c mice and maintained them on a low iron diet for approximately 4 months. The mice never developed anemia. We next performed three bleeds of approximately 500 to 600 µl (each time) over a week time period replacing the volume with 1X PBS. Values of hemoglobin and ZnPPIX/heme ratios showed, that after bleeding mice 3 times removing about 1.5 ml to 2 ml in total, we induced anemia and iron deficiency. The cut off chosen for anemia was 12 g/dl (mice with Hb lower than 12 g/dl were considered anemic) and for iron deficiency was 160 µM/M (mice with a ratio higher than 160 ZnPPIX µM/hemeM were considered iron deficient). In cases where the hemoglobin level was very close to our cut off we used the hematocrit percent to decide determine the anemic status (HCT lower than 45%); although the HCT parameter was only included in the second repetition of the experiment. Consistently, on both repetitions anemic iron deficient mice maintained their anemic status and iron deficiency after two and six weeks under a low iron diet. On the first biological repletion we observed that non-anemic mice were not rendered anemic or iron deficient by a low iron diet. In the second biological repetition, non-anemic mice with a low iron diet for 2 weeks reported a borderline value for iron deficiency whereas non-anemic mice with a low iron diet for 6 weeks showed a value higher than 160 µM/M. However, in both cases their non-anemic status was maintained (Table 3.1).

A

			Baseline level						On day of liver harvest					
	Wks	**Iron diet**	**Hb (g/dl)**	**stdev**	**Anemia**	**ZnPPIX/heme (uM/M)**	**stdev**	**Iron deficiency**	**Hb (g/dl)**	**stdev**	**Anemia**	**ZnPPIX/heme (uM/M)**	**stdev**	**Iron deficiency**
Anemic mice	**2**	Low	11.7	4.0	Yes	249.4	70.2	Yes	9.1	3.2	Yes	173.5	15.3	Yes
		Normal	10.6	2.6	Yes	229.4	29.8	Yes	13.3	1.4	No	154.2	10.7	No
		High	11.3	0.3	Yes	239.3	30.5	Yes	12.8	1.1	No	136.8	6.1	No
	6	Low	9.5	1.3	Yes	245.2	36.1	Yes	7.3	2.3	Yes	247.8	111.2	Yes
		Normal	8.4	2.2	Yes	368.0	116.8	Yes	12.8	0.5	No	131.3	3.4	No
		High	8.6	1.4	Yes	303.0	68.5	Yes	12.0	0.5	No	109.4	4.2	No
Non-anemic mice	**2**	Low	ND		ND	ND		ND	13.3	0.4	No	144.2	4.6	No
		Normal	ND		ND	ND		ND	12.9	0.4	No	148.2	6.2	No
		High	ND		ND	ND		ND	12.2	1.2	No	141.2	6.4	No
	6	Low	ND		ND	ND		ND	14.5	2.6	No	139.4	12.0	No
		Normal	ND		ND	ND		ND	15.1	1.2	No	140.6	3.6	No
		High	ND		ND	ND		ND	15.2	0.9	No	109.4	9.3	No
Control mice (not bled with normal diet)			17	1.8	No	167	10.7	No						

B

			Baseline level								On day of liver harvest							
	Wks	**Iron diet**	**HCT %**	**stdev**	**Hb (g/dl)**	**stdev**	**Anemia**	**ZnPPIX /heme (uM/M)**	**stdev**	**Iron deficiency**	**HCT %**	**stdev**	**Hb (g/dl)**	**stdev**	**Anemia**	**ZnPPIX /heme (uM/M)**	**stdev**	**Iron deficiency**
Anemic mice	**2**	Low	17.2	3.1	11.6	0.9	Yes	255.8	18.1	Yes	19.0	4.5	4.8	1.2	Yes	382.8	87.7	Yes
		High	22.5	6.0	12.5	3.0	Yes	260.3	17.2	Yes	49.2	2.9	12.5	0.6	No	143.3	5.7	No
	6	Low	19.5	7.8	11.1	3.6	Yes	339.0	55.7	Yes	22.3	12.2	5.2	3.0	Yes	507.0	141.8	Yes
		High	15.0	5.9	9.2	1.3	Yes	339.7	7.5	Yes	50.0	3.2	12.2	0.5	No	145.5	10.8	No
Non-anemic mice	**2**	Low	ND		ND		ND	ND		ND	49.9	0.9	13.0	0.2	No	161.8	7.4	Yes
		High	ND		ND		ND	ND		ND	50.7	0.5	13.4	0.3	No	156.0	10.0	No
	6	Low	ND		ND		ND	ND		ND	47.2	3.7	12.1	0.5	No	199.3	12.8	Yes
		High	ND		ND		ND	ND		ND	46.4	4.9	11.3	1.3	No	158.8	14.5	No
Control mice (not bled with normal diet)			47.4	2.4	15.6	1.0	No	152.7	11.2	No								

Table 3.1. Mice were rendered anemic and iron deficient after phlebotomies. Mice were phlebotomized 3 times removing around 1.5 to 2 mL in total. Blood samples were analyzed for hematocrit (HCT) %, Hemoglobin (Hb) and ZnPPIX/heme. Baseline level refers to the values obtained right before the change in iron diets. Day of liver harvest refers to the values obtained on the sacrifice day of mice post infection with *P. berghei* sporozoites. **A**. Corresponds to the first biological experiment considering a low, normal and high iron diet. **B.** Corresponds to the second biological repetition considering only a low and high iron diet. Balb/c control mice were never phlebotomized and received a normal iron diet. The cut off for Hb was set at 12 g/dl, for ZnPPIX/heme at 160 and for HCT % at 45.Wks = weeks; ND = no determined.

3.4.4 Only hepatic *Plasmodium* parasite loads from anemic mice maintain a strong negative relationship with increasing iron in diet

Initially we tested low, normal and high iron diets. On our first experiment (1^{st} biologic repetition) we found that after 2 weeks of feeding on iron diets, parasite load showed a negative relationship with increasing levels of iron in the diet in anemic and non-anemic mice and this negative relationship was not statistically different between anemic and non-anemic mice ($p=0.93$) (Figure 3.7A). After 6 weeks of either iron diet, parasite load maintained a negative relationship with increasing levels of iron diet only in anemic, iron replete mice. Non-anemic mice showed a weaker negative relationship between parasite load and iron diet. This difference in the relationship of parasite load and iron diet between anemic and non-anemic mice was statistically significant ($p < 0.001$) (Figure 3.7C). We repeated the experiment (2^{nd} biologic repetition), this time we only examined a low and high iron diet for 2 weeks and 6 weeks. We observed a negative relationship in the anemic iron deficient group, with increasing concentrations of iron diets. Non-anemic showed a weaker negative relationship. The difference in the effects on anemic and non-anemic mice was statistically significant ($p=0.01$) (Figure 3.7A). At 6 weeks, anemic and non-anemic groups showed a negative relationship with increasing concentrations of iron diets. The difference in the relationship between the parasite load and iron diet between anemic and non-anemic mice was not statistically significant ($p=0.20$) (Figure 3.7C). We decided to make a pooled data analysis from both repetitions. We found that after 2 weeks of iron diets, the anemic and non-anemic groups had a negative relationship with increasing concentrations of iron, although the non-anemic group showed a slightly weaker negative relationship. The difference between the two

groups was not significant (p=0.24) (Figure 3.7B). We observed a similar effect at 6 weeks on the pooled data, however this time the difference in the relationships between parasite load and iron diets between anemic and non-anemic was statistically significant (p=0.01) (Figure 3.7D).

As depicted on the bar graphs (Figure 3.8) obtained from the pooled data, we observed that the number of parasites after 2 weeks was higher in the anemic mice compared to the non-anemic mice under a low iron diet but higher in the non-anemic compared to the anemic under a high iron diet. However, differences were not statistically significant. Also, it was shown that non anemic mice had higher numbers of parasites with a low iron diet compared to a high iron diet, but this difference was not significant. Anemic mice under a low iron diet reported a higher parasite load that was statistically significant compared to anemic mice receiving a high iron diet ($p<0.001$). After six weeks, we observed again higher parasites numbers in the anemic compared to non-anemic under a low iron diet, but this difference was not significant. We found a statistically significant higher parasite load in non-anemic compared to anemic mice with a high iron diet (p=0.007). Comparison between non anemic mice with a low iron diet and a high iron diet showed that with a low iron diet the number of parasite was statistically significantly higher (p=0.03). Similarly, anemic mice with a low iron resulted in higher number parasites compared to anemic mice under a high iron diet. This difference was significant ($p<0.01$). Also, we evaluated if there was a statistical interaction between iron supplementation and anemic status at 2 and 6 weeks. We found that the increase in parasite load in non-anemic mice after an increase in iron

concentration was significantly higher than in the case of anemic mice. This interaction effect was statistically significant only at 6 weeks (p-value=0.009).

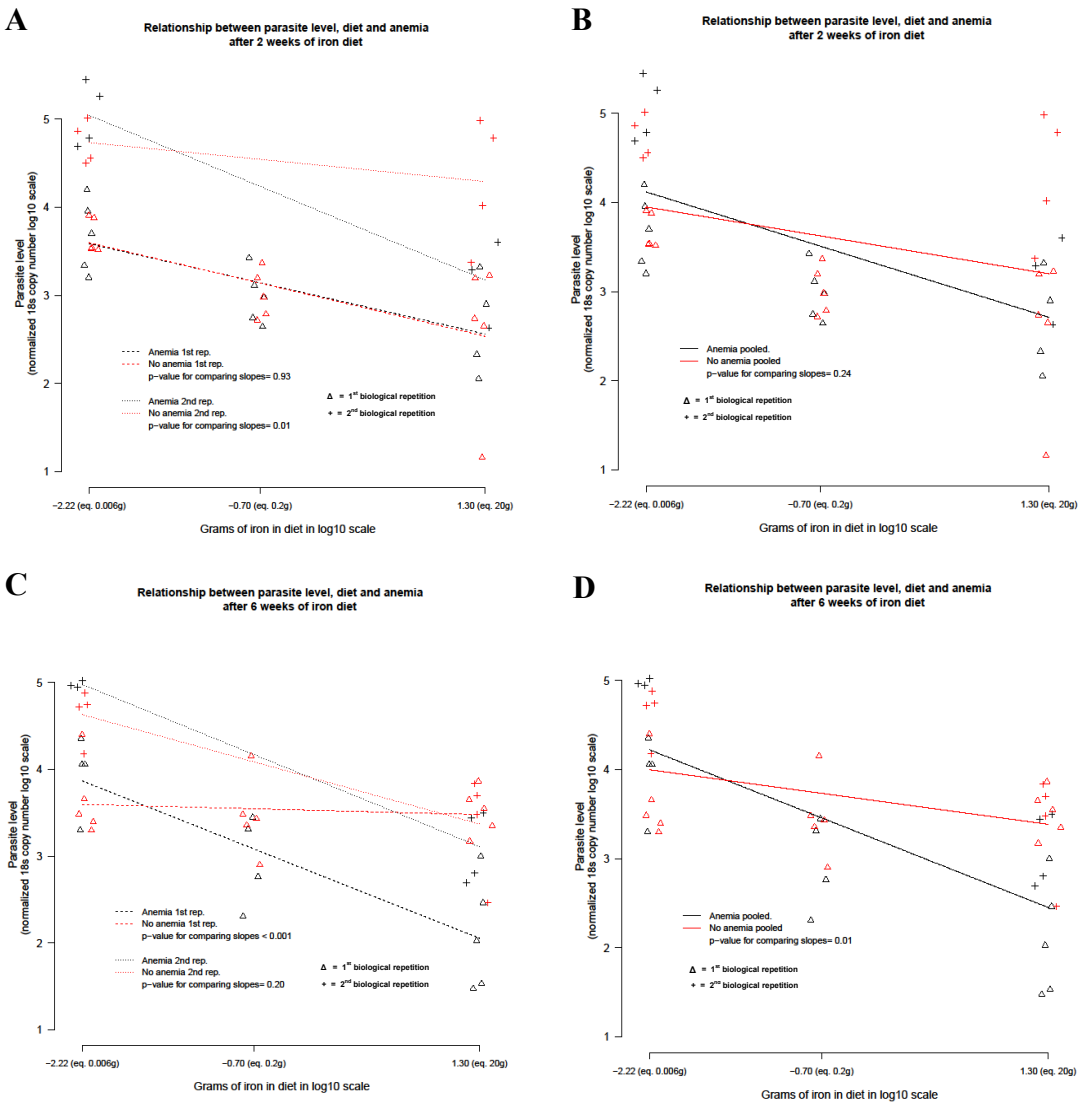


Figure 3.7. After 2 weeks of iron diets, hepatic parasite loads showed a negative relationship with increasing concentrations of iron diets in anemic and non-anemic mice whereas after 6 weeks, parasite load maintained a stronger negative relationship only in anemic mice. After bleeding, anemic iron deficient mice and non-anemic iron replete mice, in groups of five, were given iron diets with increasing concentrations of the metal. Diets were given for 2 weeks and 6 weeks *ad libitum*. Upon completion 2 or 6 week interval, anemic and non-anemic mice were infected with 15000 *P. berghei* sporozoites by tail vein injection. Forty hours after infection mice were sacrificed and their livers were collected to quantify hepatic infection level by qReal- Time PCR. **A**. Parasite level vs. Iron diets after 2 weeks showing the two repetitions. **B**. Pooled data from A. **C**. Parasite level vs. Iron diets after 6 weeks showing the two repetitions. **D**. Pooled data from C.

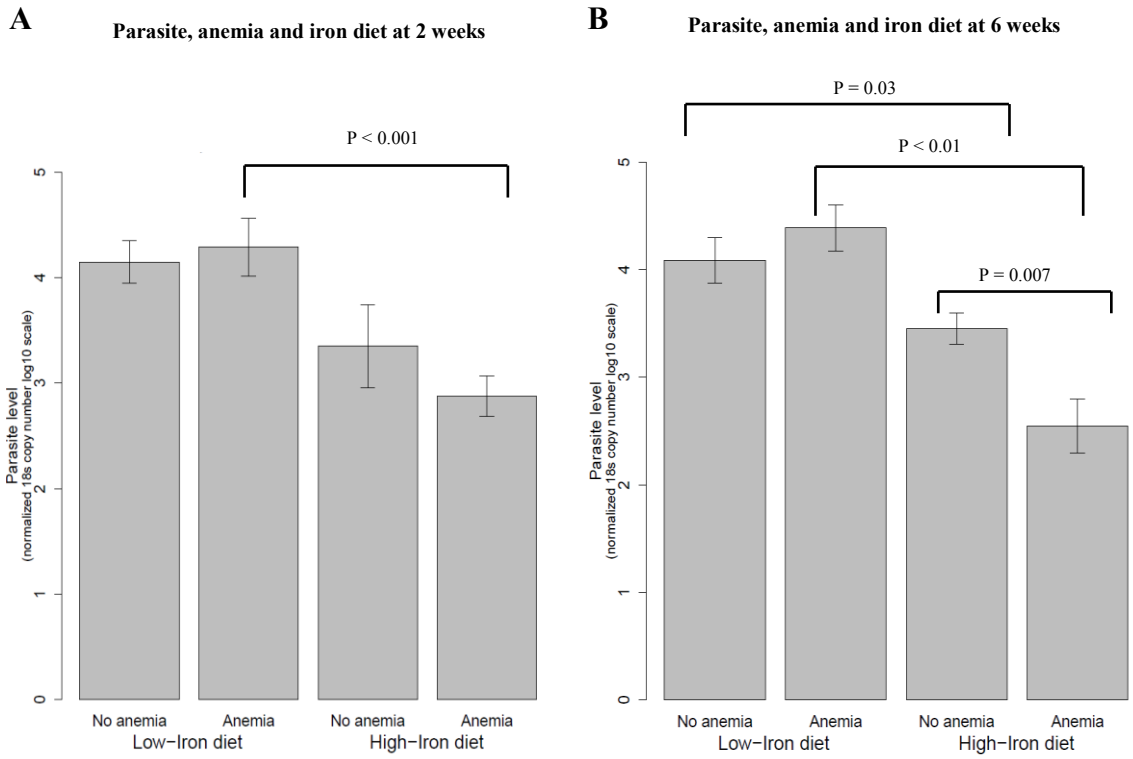


Figure 3.8. Bar graphs confirming the effect analyzed by linear regressions of the pooled data depicted on figure 3.7. Only high and low iron diets were considered for a better comparison. The trend of higher parasite load in the non-anemic mice under a high iron diet became much more evident at six weeks. **A**. Parasite load vs. iron diet after 2 weeks (based on the pooled data). **B.** Parasite load vs. iron diet after 6 weeks (based on the pooled data). P-values were obtained from a T- Student test. One analysis was made comparing the effects on parasite numbers between anemic and non-anemic mice under a low and high iron diet after 2 and 6 weeks and a second analysis was made comparing the effects on parasite numbers between low and high iron diet groups under anemic and non-anemic status after 2 and 6 weeks. Only significant values are shown.

3.4.5 Increasing iron in diet increases hepcidin levels in livers of anemic and non-anemic mice

Contrary to the trend that we found between iron diets and parasite loads, our experiments showed a positive relationship between increasing levels of iron diet and hepcidin in anemic and non-anemic groups. Increasing iron increases hepcidin. On the first repetition, this positive relationship was very distinctive when iron increases from low to normal levels and it levels off after that. This positive relationship was different between anemic and non-anemic mice when the iron diet was below normal levels ($p=0.001$ at 2 weeks and $p=0.008$ at 6 weeks) (Figures 3.9A and 3.9C). However, the significant difference was lost above normal levels of iron diet ($p=0.103$ at 2 weeks and $p=0.474$ at 6 weeks) (Figures 3.9A and 3.9C). This relationship was observed after 2 and 6 weeks of receiving the iron diet. On the second repetition, the positive relationship between hepcidin and iron diets was shown again. The positive relationship of hepcidin and iron diet when comparing anemic and non-anemic mice, was significantly different ($p<0.001$ at 2 and 6 weeks) (Figures 3.9A and 3.9C). Similarly to the parasite load, we performed a pooled data analysis from both repetitions. The inclusion of the normal iron diet on the analysis made that the positive relationship between anemic and non-anemic mice was significantly different when iron diet was below normal levels ($p=0.03$ at 2 weeks and $p=0.016$ at 6 weeks) (Figures 3.9B and 3.9D). The significant difference was lost above normal levels of iron diet ($p=0.61$ at 2 weeks and $p=0.45$ at 6 weeks) (Figures 3.9B and 3.9D). This data confirms that once iron stores are replete, hepcidin is not sensitive to further increases because it is expressed at nearly the maximum level.

As shown on the bar graphs (Figure 3.10) from the pooled data; non-anemic mice showed higher hepcidin level compared to anemic under a low iron diet, this difference was statistically significant ($p=0.006$ at 2 weeks; $p=0.001$ at weeks). Anemic mice showed higher hepcidin levels compared to non-anemic under a high iron diet; however this difference was not significant. A comparison between no anemic mice under a low iron diet and a high iron diet revealed that hepcidin levels are significantly higher after a high iron diet ($p<0.001$ at 2 weeks; $p=0.03$ at 6 weeks). Similarly, hepcidin levels were statistically significant higher in anemic mice under a high iron diet compared to a low iron diet ($p<0.01$ at 2 weeks; $p<0.01$ at 6weeks).

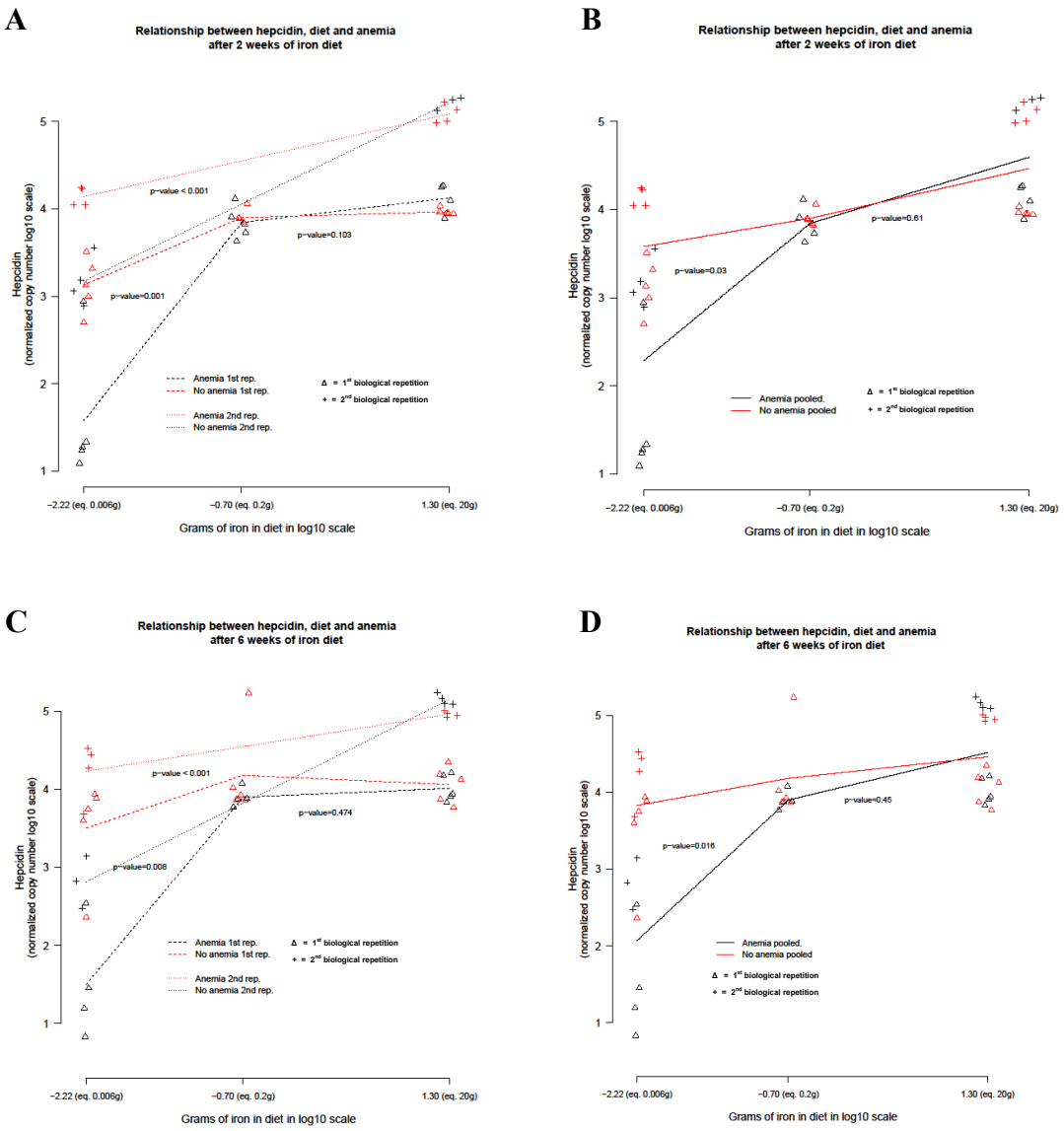


Figure 3.9. After 2 and 6 weeks of iron diets, high iron diet equals high levels of hepcidin in anemic and non-anemic mice. Anemic mice under low iron diet showed low hepcidin level. After rendering anemic mice, anemic and non-anemic mice, in groups of five, were given iron diets with increasing concentrations of the metal. Diets were given for 2 weeks and 6 weeks *ad libitum*. Upon completion with the diet time, anemic and non-anemic mice were infected with 15,000 *P. berghei* sporozoites by tail vein injection. Forty hours after infection mice were sacrificed and their livers were collected to quantify hepcidin level by qReal time PCR. **A**. Hepcidin level vs. Iron diets after 2 weeks showing the two repetitions. **B**. Pooled data from A. **C**. Hepcidin level vs. Iron diets after 6 weeks showing the two repetitions. **D**. Pooled data from **C**

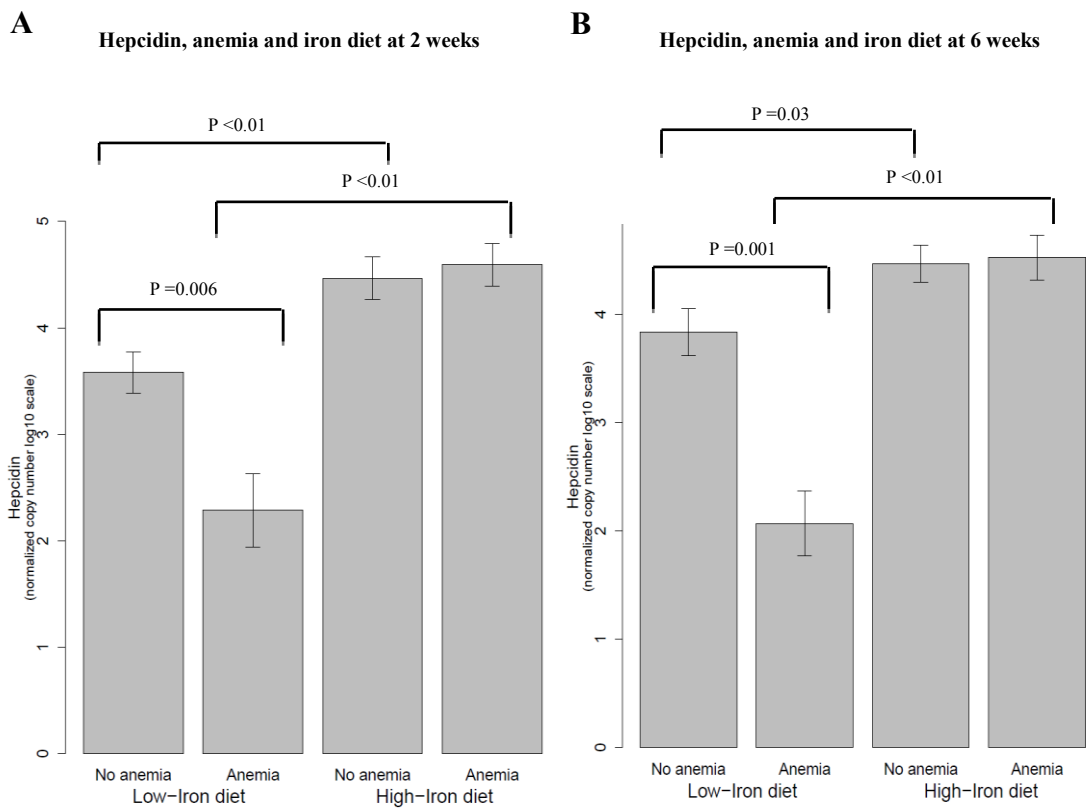


Figure 3.10. Bar graphs confirming the effect analyzed by linear regressions of the pooled data depicted on figure 3.9. Only high and low iron diets were considered for better comparison. The behavior of higher levels of hepcidin in anemic and non-anemic mice under a high iron diet was the same after 2 and 6 weeks. **A**. Hepcidin level vs. iron diet after 2 weeks (based on the pooled data). **B.** Hepcidin level vs. iron diet after 6 weeks (based on the pooled data). P-values were obtained from a T- Student test. One analysis was made comparing the effects on parasite numbers between anemic and non-anemic mice under a low and high iron diet after 2 and 6 weeks and a second analysis was made comparing the effects on parasite numbers between low and high iron diet groups under anemic and non-anemic status after 2 and 6 weeks. Only significant values are shown.

3.4.6 Nonheme iron quantification in liver and spleen correlates to iron concentration of diets

On the second repetition, after phlebotomy, iron diets and *Plasmodium* infections; we took portions of the spleens and livers to quantify tissue iron. We found that levels of nonheme iron were higher in the livers and spleens of mice that received a high iron diet regardless of their anemic status and time with the diets and that there is a drop of iron between 2 and 6 weeks. Anemic mice with a low iron diet showed the lowest levels of iron in the spleen (Table 3.3).

			Liver		Spleen	
	Weeks	**Iron diet**	**Total non heme iron mean (ug/g)**	**stdev**	**Total non heme iron mean (ug/g)**	**stdev**
Anemic mice	**2**	Low	18.6	2.4	30.3	11.1
		High	1040.3	167.2	393.9	83.2
	6	Low	38.9	12.1	16.7	5.0
		High	312.1	90.0	745.6	89.8
Non-anemic mice	**2**	Low	50.2	15.3	130.5	12.7
		High	798.3	107.9	485.4	61.7
	6	Low	51.0	18.1	127.2	71.2
		High	285.4	68.3	1085.3	300.0

Table 3.4. Nonheme iron on spleens and livers from anemic and non-anemic mice under low and high iron diet. Portions of spleens and livers were taken on day of liver harvest, 36 hours post infection with 15,000 *P. berghei* sporozoites. Tissues were digested in acid solution for 50 h at 65ºC and iron was quantified with a chromogen reagent containing bathophenanthroline sulfonate at 535 nm. Tissues quantified correspond to the animals from the second repetition only. Measurements were expressed as micrograms of iron per gram of tissue.

3.5 Discussion

Different studies have shown the importance of iron for blood and hepatic stages of *Plasmodium*. One recent reference highlighted the importance of iron on erythrocytes for the parasite (personal communication by Carla Cerami Hand). Similarly, we studied the importance of iron in hepatocytes for hepatic stage development.

The use of transgenic mice overexpressing hepcidin (Tg+) and hemoglobin deficient mice (hbd) were chosen over other transgenic anemic mice on the basis of different iron deficient compartments, either in the liver or the erythroid compartment. Tg+ mice have normal to low erythroid iron, higher iron in macrophages and low iron in hepatocytes whereas hbd mice have very low erythrocyte iron, but unchanged hepatocyte iron stores. Based on these conditions we thought that if iron location was important for the parasite, Tg+ mice would have a decrease in liver stage malaria and on the contrary hbd mice would have unaffected liver stage infection compared to control mice. Our results supported these hypotheses and showed that iron compartmentalization is indeed important during liver stage infection.

Facial bleed was a good method to induce anemia in mice compared to diet induced anemia (tried at the beginning without success, data not shown). Besides anemia, phlebotomy can also induce hypoxia, increased Epo and erythropoiesis and decreased hepcidin mRNA[159]. We were able to maintain anemia and iron deficiency for both 2 and 6 weeks of the low iron diet.

Mice receiving a standard diet in the laboratory need at least 2 weeks of an iron deficient diet to decrease the basal hepcidin expression, when diet is changed to a higher

iron diet, the increased response begins at 24 h and is maintained during all the time mice are fed with the same diet[149]. This effect was seen in our anemic mice after 2 weeks and 6 weeks of high iron diets. Once hepcidin is produced, it causes an eventual redistribution of iron from hepatocytes to macrophages. With less bioavailable iron within hepatocytes it is expected that the development of parasite may be compromised. In our study we did find a direct increasing correlation between higher hepcidin and high iron diets with both the 2 and 6 week intervals.

The iron chelator FBS0701 showed a dose – response effect on the infection level and was able to reduce significantly the parasite load with a high concentration (1000 mg/kg). Primaquine, a known antimalarial to treat hypnozoites (stage on hepatocytes) showed a synergistic effect with FBS0701. This suggests that the new iron chelator could be used in a combination therapy to treat not only blood stages but also liver stages. We found not statistical significance between the different dose schedules, although we found a trend of reduction in parasite load when drug was given on a single dose post infection (capable of curing mice with a blood stage infection[158]) and when it was given -1, 0,+1days. The spike on day 0 disappears once outlayer values are removed. The liver *P. berghei* growth takes approximately 48 hours in a mouse. Considering that parasites begin to replicate at 24 hours and the plasma concentration of FBS0701 at 1 hour is 17.5 μM and at 24 h is around 0.1 μM it would explain why FBS0701 can be apparently more effective at the mentioned schedules: iron chelation to deprive from iron enzymes involved in replication. Under this idea we should see a similar effect in the last schedule (0,+1), however this was not the case. We suggest a repetition of the experiment to see if data are consistent.

In our experiments we used about ten times the sporozoite inoculum given by mosquitoes for the qReal-Time PCR or bioluminescent imaging. We have not proven but predict that the effect of FBS0701 possibly may prevent blood stage infection with mosquito inoculum of sporozoites which deliver a few hundred sporozoites rather than the experimental doses of 10,000 used here. A single human dose of FBS0701 is predicted to remove about 20-30 mg of iron over a 24 hour period. This amount should not affect total iron status in a short 3-5 day treatment regimen for malaria. Critically FBS0701 perturbs bioavailable iron for the liver and erythrocyte *Plasmodium* parasite.

A collaborator, Carla Cerami Hand had focused on human *P. falciparum* response to iron supplementation in anemic and non-anemic individuals. We focused on liver stage malaria quantification in liver stage malaria mouse model. The mice received iron-supplemented diets and at baseline were both anemic and iron deficient as measured by elevated ZnPPIX to heme ratios or at baseline were neither anemic nor iron deficient. Our results from the two biologic replicate experiments showed that parasite liver levels in anemic mice had a strong negative correlation with high iron diets after 2 and 6 weeks. The low parasite liver levels were inversely correlated with a recent increase in hepcidin values. However, at 2 weeks we observed only weak negative relationship with the iron diets in the non-anemic group (this may be explained by the increased variability in non-anemic mice on high diet). Also, we confirmed that the difference in parasite numbers between the anemic and non-anemic groups receiving a high iron diet was not significant. Probably, during this time, this effect was caused by the increased expression of hepcidin as a response to increasing concentrations of iron in the serum and regardless of the iron stores on iron-replete mice (non-anemic). After 6 weeks of iron diets, results on non-

anemic mice showed that the effect of endogenous iron stores along with iron supplementation was more predominant than the action caused by endogenous hepcidin (induced by iron loading). We observed higher level of liver parasites with increasing iron diets. Therefore, we were able to partially replicate the findings in Pemba, that there was an increased incidence and severity to disease in iron replete children, after 6 weeks of diet, but began to see this trend at 2 weeks. Also, the study published by *Portugal et al*, showed that when non-anemic mice received 3 doses of ferric ammonium citrate 24 h and 4 h before and 20 h post infection with sporozoites, they had a significant higher parasite load in the liver compared to control mice[9]. Here, the time of iron supplementation was much shorter than in our studies, but probably because mice were already iron replete and they received 3 continuous doses of ferric ammonium citrate at 250mg/kg they had the same effect we observed clearly after 6 weeks of diet.

Portugal et al. demonstrated the importance of iron compartmentalization for parasite development mediated by hepcidin[9]. We have also demonstrated the importance of iron in transgenic mouse models of disordered iron homeostasis (Tg+ and hbd mice) and on our phlebotomized anemic mice after 2 weeks of iron supplementation, and the correlation between hepdicin and diets and parasite loads. In general, we postulate that in the hepcidin-iron-infection axis, hepcidin plays a main role acutely whereas the presence of iron itself (through supplementation in this case) is predominant over time. A recent increase in hepcidin will limit bioavailable iron in the liver and decrease parasite liver load. The same hepcidin level after 6 weeks does not maintain low bioavailable iron in the face of iron supplementation. With iron supplementation after a few weeks we see an increase in parasite liver levels. However, this may not be the only factor influencing our

results. The quantification of nonheme iron on the spleen and liver tissues showed that liver iron is actually dropping between 2 and 6 weeks. This may be a "first pass" effect. Blood flows from the serosal side of the duodenum to the liver first. There, the iron is recovered and stored by the liver, but eventually gets loaded into new red cells. When they are phagocytized by macrophages of the spleen, then spleen iron eventually increases. The fact that anemic mice have the lowest spleen iron levels correlates with the use of splenic iron for stress erythropoiesis in these mice. Also, the spleen iron levels correlate well with the increasing levels of hepcidin found when giving a high iron diet (therefore increasing the levels iron in the serum). The question is why if the iron levels on the livers of anemic mice are higher or very close to those of non-anemic mice, and considering that hepcidin is also being increased simultaneously, parasites loads on those anemic mice are not high. We believe that in the context of non anemia (or iron replete individuals) along with iron supplementation, exogenous iron induces the increase in hepcidin, which would target ferroportin present on Kuffer cells and hepatocytes inducing the degradation of the iron exporter, causing the accumulation of iron within those cells. But in the context of anemia and iron supplementation, the responsiveness of ferroportin in the hepatocytes would be reduced allowing the export of iron from those cells. The heme iron quantification assay did not allow us to discriminate iron from macrophages or hepatocytes.

There are several studies that have evaluated the importance of iron by itself and the efforts of parasites to obtain the metal. *Alburquerque et al*. have shown that parasites can remodel infected cells to facilitate their development. Hepatoma cells infected with *Plasmodium* showed a decrease of ferroportin and an increase of DMT-1 to favor

accumulation of iron in hepatocytes[160]. *Clark et al*. showed that, without considering the effect of hepcidin on iron regulation, deficient anemic patients who received iron supplementation increased their erythropoiesis and replaced their microcytic iron-deficient RBCs with new normocytic and iron-replete RBCs. The change in RBC physiology and population would favor blood stage infection. Lastly, this same group proved that parasitized RBCs increase their labile iron pool along with the parasite life cycle and needs. They confirmed that parasites can even access iron sources from host serum[161].

Therefore, our hypothesis is that hepcidin reaches a plateau causing a redistribution of iron and subsequently influences the decrease of parasites and, once reached that level, hepcidin effects become less significant in the presence of a continuous inducer of parasite growth like iron supplementation.

In conclusion, based on the importance of iron for the parasite and the human host, it would seem more reasonable to put in practice the recommendation of iron supplementation along with preventive therapy in malaria endemic areas.

CHAPTER 4

EFFECT OF ANTIMALARIAL IRON CHELATOR FBS0701 ON *PLASMODIUM FALCIPARUM* MOSQUITO STAGES

4.1 ABSTRACT

Current efforts to fight against malaria include both antimalarial drugs and vaccines. Iron chelators have been studied as promising drugs due to its antimalarial properties against different stages of the parasite *in vitro* and *in vivo*. The new iron chelator FBS0701, already tested in *Plasmodium* blood stages, was shown to affect development of early, not late *P. falciparum* gametocytes and to cure *P. yoelii* infected mice. Here we tested the effect of FBS0701 on late stage *P. falciparum* gametocyte infectivity to mosquitoes. Incubation of mature gametocytes for 24, 48 and 72 hours with increasing concentrations of FBS0701 (12.5-50 μM) resulted in a significant reduction in mosquito infectivity measured by the oocyst number per mosquito and up to 96% reduction in prevalence (% infected mosquitoes). This reduction in mosquito infectivity was due to mortality of gametocytes as shown by propidium iodide staining and exflagellation assays despite minimum morphologic changes by Giemsa blood film. Pre incubation of FBS0701 with ferric chloride restored the gametocyte infectivity showing that the inhibitory effect of FBS0701 was a result of iron chelation.

Deferoxamine, another iron chelator also reduced gametocyte infectivity but to a lesser extent. Finally, co-feeding the gametocytes together with FBS0701 to mosquitoes did not significantly change the oocyst numbers, suggesting that iron chelation by FBS0701 is not detrimental for the sexual stages of the parasite inside the mosquito midgut. These results show the importance of gametocyte iron metabolism as a potential target to develop new transmission-blocking strategies.

4.2 INTRODUCTION

Malaria remains a major health problem in tropical and subtropical regions. As estimated by WHO in 2012 there were 207 million infected people and around 627 000 deaths in the world[162]. One of the main problems to control malaria transmission is the detection and treatment of gametocyte carriers. These include people treated for the asexual stages of malaria that might have circulating gametocytes for up to a month after the treatment and asymptomatic malaria patients that without treatment could have circulating gametocytes for many months[163]. Current efforts to control malaria transmission include the improvement of methods to detect gametocyte carriers as well as the development of transmission-blocking drugs and vaccines which aim to interrupt parasite reproduction and development in the vector.

The quinolines like chloroquine and quinine have minimal effects on mature sexual stages of *P. falciparum* and although patients are cured, they still can transmit to mosquitoes[164,165,166].

Studies show that quinine and mepacrine increase the number of gametocytes and therefore transmission to mosquitoes[167]. Chloroquine and quinine affect early gametocytes by disrupting hemoglobin degradation whereas atovaquone inhibits cytochrome b, important for the mitochondrial membrane potential[164]. None of these drugs affect late gametocyte stages[167,168]. Artemisinin-based combination therapy (ACT) reduces gametocytes circulating in the blood by targeting early gametocytes, although transmission can still occur after treatment[169,170]. Primaquine, the only drug currently used to target mature *P. falciparum* gametocytes[171] and other 8-aminoquinolines can kill

mature *P. falciparum* gametocytes[172], however, they have poor activity against asexual stages and might target mitochondrial function[173]. Also, the active metabolite of primaquine has safety issues in people with glucose-6-phosphate dehydrogenase deficiency leading to hemolytic anemia[174]. A new drug-screening study against *Plasmodium* showed that, 3 new agents in development: NPC1161B, OZ277 and methylene blue, reduce gametocyte viability by at least 50%[175].

Iron, an important element for the metabolism of all living organisms, has been considered as a target to treat malaria for the past few decades. Experimental evidence indicates a low molecular weight chelatable non-ferritin pool in erythrocytes, which provides bioavailable iron for *Plasmodium*[176]. Studies show the inhibition of parasites by iron chelators in mice and humans[176,177]. During gametocytogenesis, the tricarboxylic acid cycle becomes more active[178,179] mainly to produce succinyl Co-A for heme biosynthesis instead of the full oxidation of glucose[180]. In *P. falciparum*, cytochrome b, which binds two heme molecule groups, has an increased expression of several fold in sexual stages compared to asexual stages[181]. In addition, mRNAs of three of the six genes involved in heme biosynthesis are upregulated during gametocytogenesis[182].

Plasmodium growth in culture is inhibited by iron chelators, with a greater effect on the trophozoite stage[184,98]. In addition, iron chelators inhibit liver stage malaria and iron supplementation increases the number of merozoites released from the liver[176]. Therefore, iron chelation and iron deficiency can inhibit *Plasmodium* growth *in vivo*.

FBS0701 is a new iron chelator that recently completed a Phase 2 clinical trial to treat transfusional iron overload[111]. FBS0701 can be administered as a single daily dose

because of its favorable absorption and pharmacokinetic properties compared to the other iron chelators- deferoxamine and deferiprone[110]. FBS0701 was recently shown to inhibit early gametocyte stages[158]. Although iron chelation is a good strategy to target malaria there is need for the development of a more suitable and safer drug.

In general, the metabolism of *Plasmodium* gametocytes and especially their iron requirements have not been explored in detail. In this study, we tested the activity of FBS0701 as a potential transmission-blocking drug on *P. falciparum* sexual stages.

4.3 METHODS

4.3.1 Materials

FBS0701 was obtained from Aptuit, Kansas City, LLC under good manufacturing practice. *P. falciparum* NF54 cultures were obtained from Johns Hopkins School of Public Health Core Facility.

4.3.2 Drug treatment

Drug assays were performed in 6-well plates by adding dilutions of FBS0701 (3 µM - 100 µM) and deferoxamine (DFO) (Sigma, St. Louis, MO) at 12.5 µM and 25 µM. Drugs were added to fifteen day old *P. falciparum* NF54W gametocytes which are considered late stage. Cultures were maintained in complete RPMI-1640 with glutamine (Corning cell gro, Manassas, VA), along with HEPES, 10% human serum and 50 mg/ml

hypoxanthine without antibiotics. Parasitemia was maintained at 2.5% and 4% hematocrit in a gassed jar (5%O_2, 5% CO_2 and 90% N_2) at 37ºC for 24, 48h or 72h. Control parasites were incubated with complete RPMI media without antibiotics. Medium was replaced daily with new FBS0701 diluted in RPMI without the addition of erythrocytes.

The same methodology was followed to incubate cultures with ferric chloride at 2.5 µM, 12.5 µM and 50 µM alone or in combination with FBS0701 at 25 µM for 24 h.

4.3.3 Membrane feeding assays

Cultures were centrifuged at 108x g for 4 minutes. Pellets were resuspended with human serum and erythrocytes to a gametocytemia of 0.1% and 50% hematocrit. Gametocytes were fed to 6 day old sucrose-starved female *An. gambiae* mosquitoes through a membrane feeder (glass-made with an outer chamber for circulating 37ºC water and with a piece of parafilm covering the lower portion of the feeder).

After feeding, fed mosquitoes were sorted and maintained at 27ºC for 7 days until dissection of their midguts. Midguts were stained with 0.1% mercurochrome and oocyst number was used to evaluate the effect of drugs on the parasites.

4.3.4 Exflagellation assays

Ten microliters of the gametocyte culture from each treatment were pelleted and resuspended in 10 µl of exflagellation media (RPMI 1640 medium (Corning cell gro,

Manassas, VA) with 25 mM Hepes, 2 mM glutamine, 50 mg/ml hypoxanthine, 2 g sodium bicarbonate, 1 µM xanthurenic acid and 20% human serum). Exflagellation reactions were incubated for 15 minutes at room temperature and then loaded into an Improved Neubauer chamber (0.1 mm deep, Reichert, Buffalo, NY) for estimation of exflagellations and erythrocytes per µl of culture. Results were reported as the number of exflagellations per 10^6 RBCs.

4.3.5 Viability assays

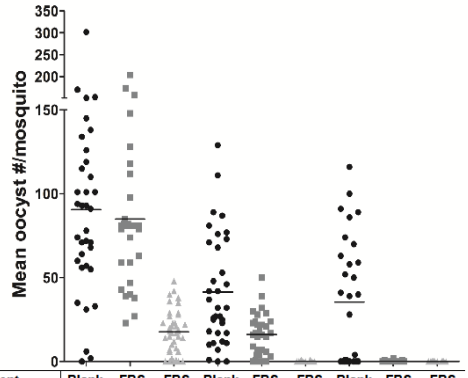
After incubation with FBS0701 at 25 µM for 48 h as described above, 100 µl of that culture were mixed with 10 µl of propidium iodide (PI) (100 ug/ml) (Sigma, St Louis, MO) and incubated for 10 min at 37ºC. Then 10 µl of the stained sample were pelleted by a quick spin at max speed and the pellet was resuspended with 10 µl of 37ºC 1X PBS and placed immediately on a slide to be observed by fluorescent microscopy (Nikon upright 90i microscope). Viable gametocytes do not stain with PI while dead gametocytes become permeable and stain with PI. A total of 100 gametocytes were analyzed for each treatment.

4.4 RESULTS

4.4.1 FBS0701 affects *P. falciparum* gametocyte infectivity

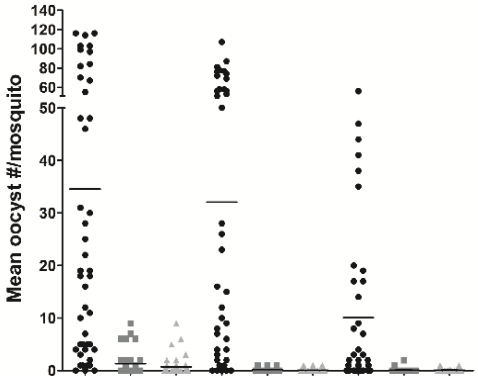
To analyze the effect of FBS0701 on *P. falciparum* gametocyte infectivity to mosquitoes, mature gametocytes were incubated with different concentrations of FBS0701 for 24, 48 and 72 hrs and then fed to *An. gambiae* mosquitoes. To measure gametocyte viability, oocyst numbers were determined on mosquito midguts dissected 7 days post infection (dpi). We found a significant reduction in the number of oocysts that was dependent on the dose and time of incubation of *P. falciparum* gametocytes ($p<0.001$ at 48 h and 72 h) (Figure 4.1A and B). A hundred percent of oocyst inhibition was found from a dose of 25 μM FBS0701 at 48 h (Figure 4.1C). The prevalence confirms the dose-response pattern. We observed around 60% reduction in prevalence with a dose of 50 μM at 24 h and reduction of more than 80% with 25 μM at 48 h and 72 h (Figure 4.1D). Thin blood smears of the cultures before membrane feeding confirmed that gametocytes were actually present and morphologically intact before infecting the mosquitoes. We did not find large reduction in the number of gametocytes regardless of the incubation time or FBS0701 dose (Figure 4.1E).

A



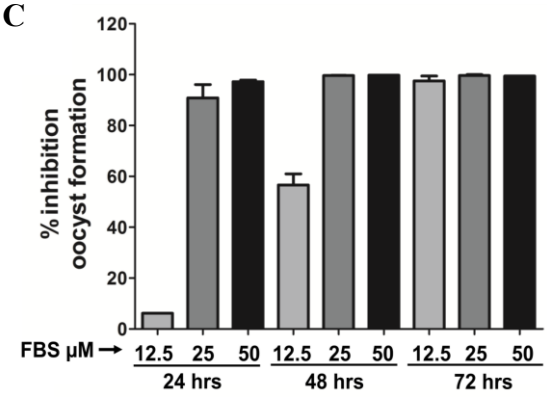
Treatment	Blank	FBS	FBS	Blank	FBS	FBS	Blank	FBS	FBS
Concentration (µM)	0	12.5	25.0	0	12.5	25.0	0	12.5	25.0
Treatment Period (hrs)	24	24	24	48	48	48	72	72	72
Mean oocyst	90.6	85.0	17.8	41.5	16.2	0.11	35.4	0.19	0
% inhibition	-	**6.19**	**80.4**	-	**61.0**	**99.7**	-	**99.5**	**100**
Prevalence	97.1	100	87.5	94.3	87.5	10.7	63.3	14.8	0
% reduction Prev.	-	**0**	**9.93**	-	**7.2**	**88.6**	-	**76.6**	**100**
N	35	29	40	35	32	28	30	27	18
P-value	-	NS	<0.001	-	<0.001	<0.001	-	<0.001	<0.001

B



Treatment	Blank	FBS	FBS	Blank	FBS	FBS	Blank	FBS	FBS
Concentration (µM)	0	25	50	0	25	50	0	25	50
Treatment Period (hrs)	24	24	24	48	48	48	72	72	72
Mean oocyst	34.6	1.31	0.73	32.1	0.17	0.07	10.1	0.07	0.05
% inhibition	-	**96.2**	**97.9**	-	**99.5**	**99.8**	-	**99.3**	**99.5**
Prevalence	**93.3**	**52.1**	**26.7**	**87.5**	**17.1**	**6.7**	**61.5**	**4.8**	**5.4**
N	45	48	45	40	41	45	39	42	37
P-value	-	<0.001	<0.001	-	<0.001	<0.001	-	<0.001	<0.001

C



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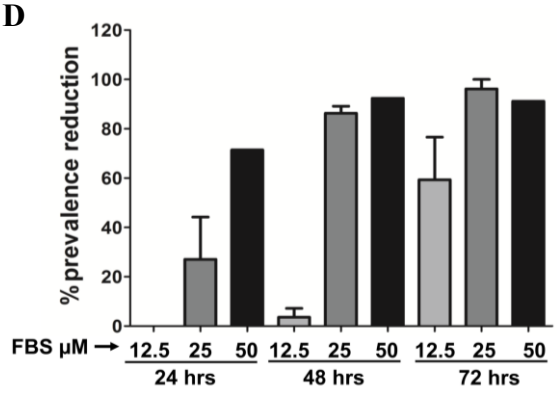

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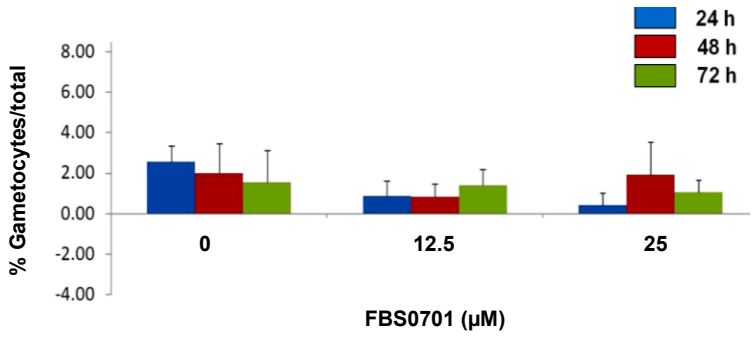
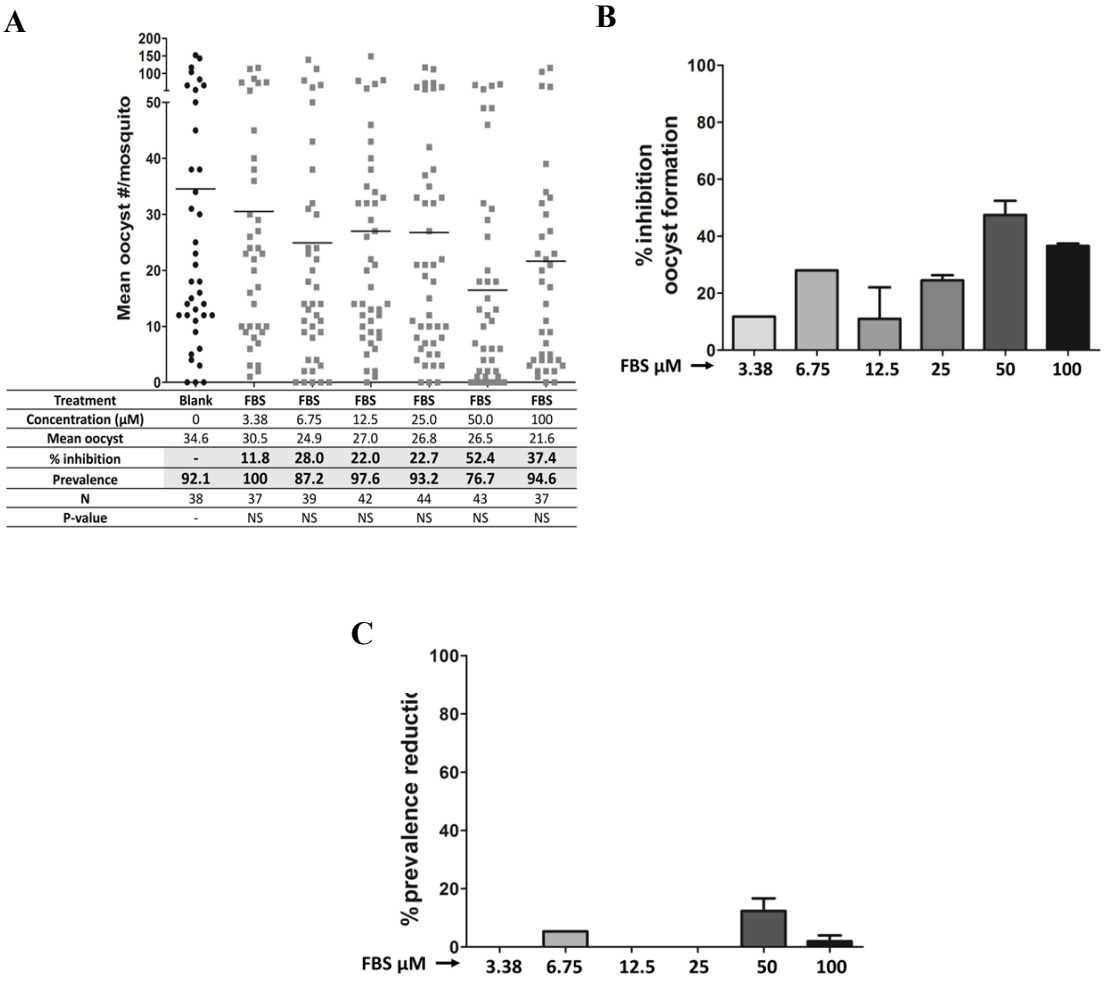


Figure 4.1: Incubation of *P. falciparum* gametocytes with FBS0701 inhibits development in the mosquito. To analyze the effect of FBS0701 (or FBS) on malaria gametocytes, *P. falciparum* mature gametocytes were incubated with different concentrations of FBS0701 for 12, 48 and 72 hours. Gametocytes were fed to *An. gambiae* mosquitoes and oocyst numbers were determined in mosquito midguts 7 days post infection. **A-B.** Representative experiments. N= number of mosquitoes. Percent inhibition = [(mean oocyst # of control mosquitoes - mean oocyst # of experimental mosquitoes) / mean oocyst # of control mosquitoes] x 100. Prevalence = percentage of infected mosquitoes, Percentage reduction in prevalence = [(prevalence of control mosquitoes – prevalence of experimental mosquitoes) / prevalence of control mosquitoes] x 100. Significance of differences between the mean oocyst number of FBS0701 treatments compared to blank control were determined by One way Anova with Bonferroni's Multiple Comparison Test. **C.** Percent inhibition of oocyst formation. Bars represent the average % inhibition of oocyst formation from independent experiments shown in Table A1 (pooled data). Error bars represent the standard error of the mean. **D**. Percent reduction in prevalence. Bars represent the average % reduction in prevalence (% infected mosquitoes) compared to the blank control, from independent experiments shown in Table A1 (pooled data). Error bars represent the standard error of the mean. **E.** Percent of gametocytes present before membrane feeding. Smears were made from each FBS0701 treated culture and gametocytes were counted by microscopy upon Giemsa staining. Data was obtained from one biological replicate. Error bars represent the standard error of the mean.

4.4.2 FBS0701 does not affect *P. falciparum* mosquito midgut stages

A method of testing if the drug was actually affecting mosquito stages was adding drug to the gametocyte cultures and proceed immediately to the membrane feeding without incubation. We found that FBS0701 does not affect mosquito stages revealed by non-significant difference in the oocyst number (Figure 4.2A). The highest percent of inhibition was obtained with a dose of 50 μM but did not surpass 60 %, whereas at that same concentration after 24 h of incubation we observed around 100% of oocyst inhibition (Figure 4.1B). Similarly, the reduction in prevalence at 50 μM was only around 20% compared to 70% achieved with the same dose after 24 h (Figure 4.1C).

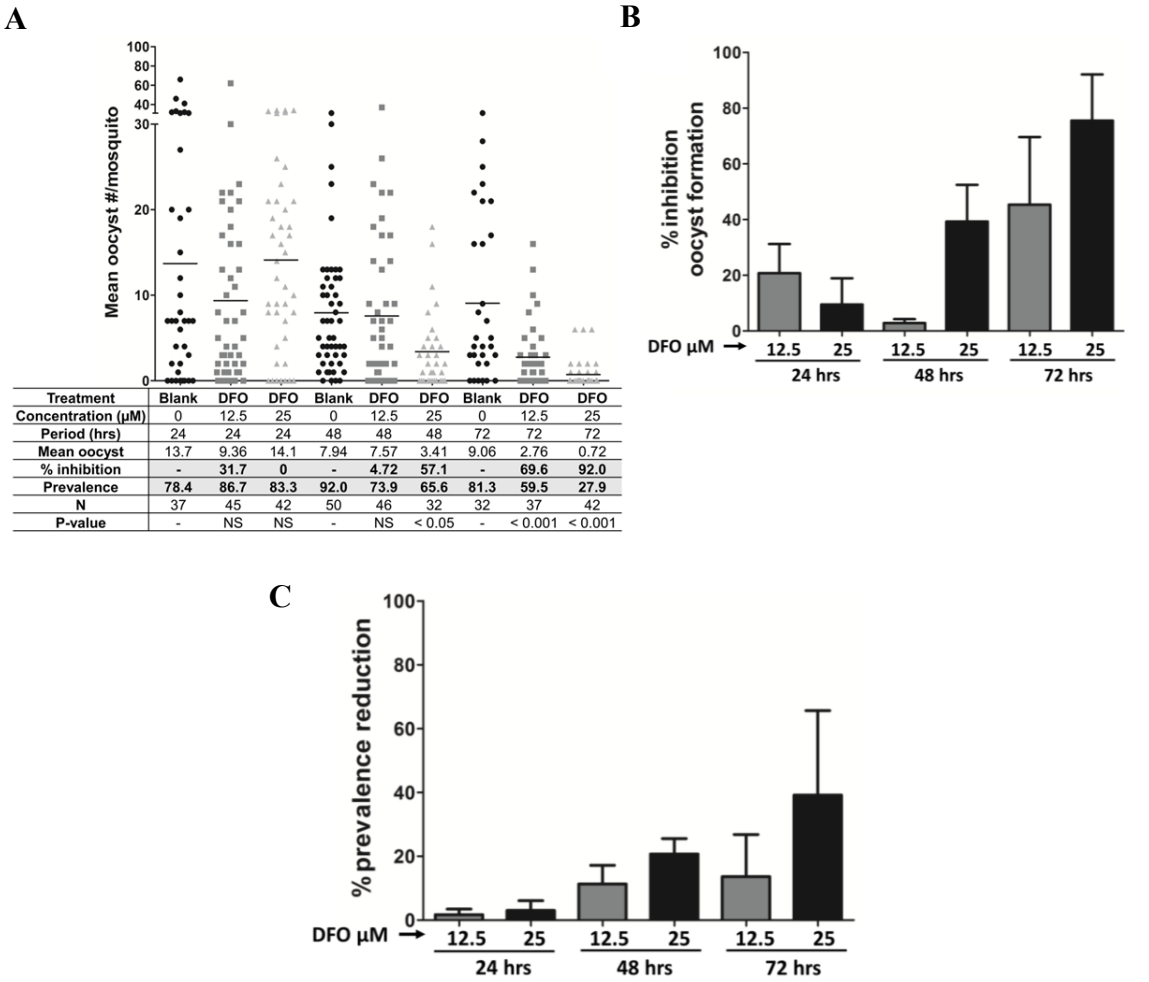


Treatment	Blank	FBS	FBS	FBS	FBS	FBS	FBS
Concentration (µM)	0	3.38	6.75	12.5	25.0	50.0	100
Mean oocyst	34.6	30.5	24.9	27.0	26.8	26.5	21.6
% inhibition	-	11.8	28.0	22.0	22.7	52.4	37.4
Prevalence	92.1	100	87.2	97.6	93.2	76.7	94.6
N	38	37	39	42	44	43	37
P-value	-	NS	NS	NS	NS	NS	NS

Figure 4.2. FBS0701 does not affect *Plasmodium* sexual development in the mosquito midgut lumen. To analyze the effect FBS0701 (or FBS) on sexual stages of *Plasmodium* (gametes, zygotes and ookinetes), *P. falciparum* gametocytes were fed to *An. gambiae* mosquitoes in the presence of different concentrations of FBS0701. Oocyst numbers were determined in mosquito midgets 7 days post infection. **A.** Representative experiment. N= number of mosquitoes. Percent inhibition = [(mean oocyst # of control mosquitoes - mean oocyst # of experimental mosquitoes) / mean oocyst # of control mosquitoes] x 100. Prevalence = percentage of infected mosquitoes, Percentage reduction in prevalence = [(prevalence of control mosquitoes – prevalence of experimental mosquitoes) / prevalence of control mosquitoes] x 100. Significance of differences between the mean oocyst number of FBS0701 treatments compared to blank control were determined by One way Anova with Bonferroni's Multiple Comparison . NS= not significant. **B.** Percent inhibition of oocyst formation. Bars represent the average % inhibition of oocyst formation from independent experiments shown in Table A4 (pooled data). Error bars represent the standard error of the mean. **C.** Percent reduction in prevalence. Bars represent the average % reduction in prevalence (% infected mosquitoes) compared to the blank control, from independent experiments shown in Table A4 (pooled data). Error bars represent the standard error of the mean.

4.4.3 Deferoxamine effects on mature *P. falciparum* gametocyte infectivity are milder compared to FBS0701

Next, we tested the effect of DFO, another iron chelator known to have antimalarial activity against *P. falciparum*. We used 12.5 μM and 25 μM and the same incubation times as for FBS0701. We found that DFO induces a significant oocyst decrease ($p< 0.05$ and $p< 0.001$ at 48 h and 72 h respectively) in a similar dose response behavior as FBS0701 (Figure 4.3A). However, the percent of oocyst inhibition never reached to 100 even at 25 μM after 72 h (Figure 4.3B). The highest reduction in prevalence observed was only around 40% with a dose of 25 μM after 72h (Figure 4.3C).

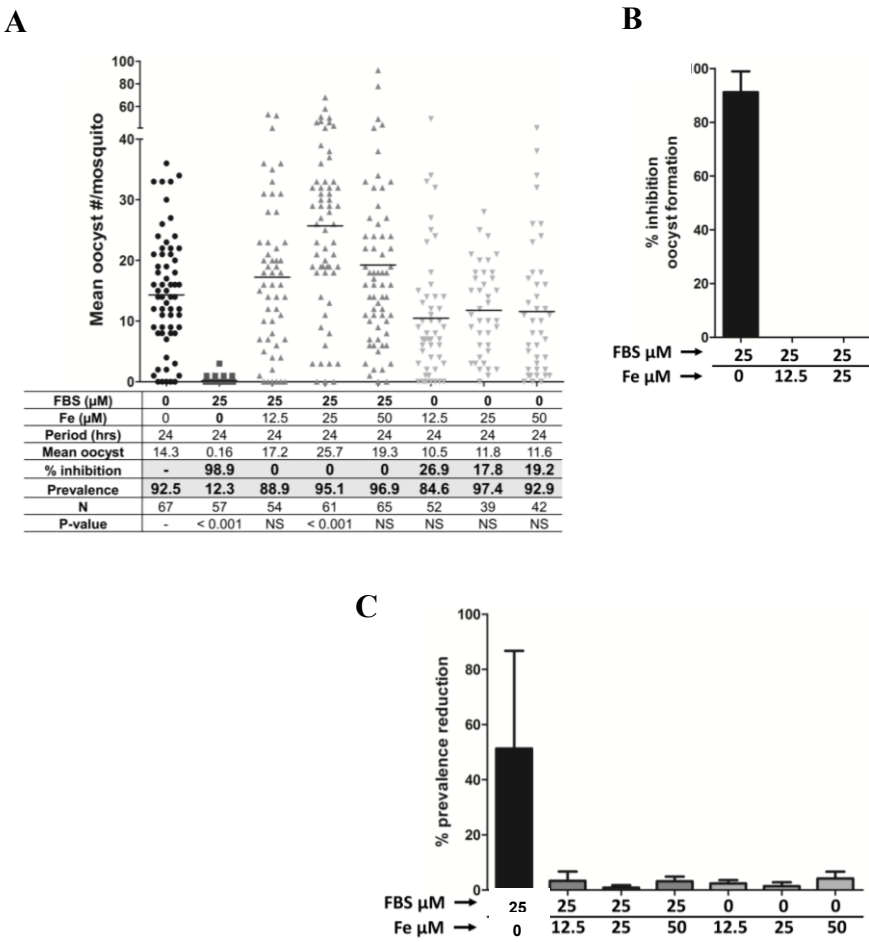


Treatment	Blank	DFO	DFO	Blank	DFO	DFO	Blank	DFO	DFO
Concentration (µM)	0	12.5	25	0	12.5	25	0	12.5	25
Period (hrs)	24	24	24	48	48	48	72	72	72
Mean oocyst	13.7	9.36	14.1	7.94	7.57	3.41	9.06	2.76	0.72
% inhibition	-	**31.7**	**0**	-	**4.72**	**57.1**	-	**69.6**	**92.0**
Prevalence	**78.4**	**86.7**	**83.3**	**92.0**	**73.9**	**65.6**	**81.3**	**59.5**	**27.9**
N	37	45	42	50	46	32	32	37	42
P-value	-	NS	NS	-	NS	< 0.05	-	< 0.001	< 0.001

Figure 4.3: Incubation of *P. falciparum* gametocytes with DFO inhibits development in the mosquito to a lesser extent. To analyze the effect of DFO on malaria gametocytes, *P. falciparum* mature gametocytes were incubated with different concentrations of FBS0701 for 12, 48 and 72 hours. Gametocytes were fed to *An. gambiae* mosquitoes and oocyst numbers were determined in mosquito midguts 7 days post infection. **A.** Representative experiment. N= number of mosquitoes. Percent inhibition = [(mean oocyst # of control mosquitoes - mean oocyst # of experimental mosquitoes) / mean oocyst # of control mosquitoes] x 100. Prevalence = percentage of infected mosquitoes, Percentage reduction in prevalence = [(prevalence of control mosquitoes – prevalence of experimental mosquitoes) / prevalence of control mosquitoes] x 100. Significance of differences between the mean oocyst number of FBS0701 treatments compared to blank control were determined by One way Anova with Bonferroni's Multiple Comparison Test. **B.** Percent inhibition of oocyst formation. Bars represent the average % inhibition of oocyst formation from independent experiments shown in Table A2 (pooled data). Error bars represent the standard error of the mean. **C**. Percent reduction in prevalence. Bars represent the average % reduction in prevalence (% infected mosquitoes) compared to the blank control, from independent experiments shown in Table A2 (pooled data). Error bars represent the standard error of the mean.

4.4.4 Iron blocks FBS0701 and restores gametocyte fertility

The loss of fertility of *P. falciparum* gametocytes incubated with FBS0701 is presumably induced by iron chelation. To test this hypothesis, FBS0701 (25 μM) was pre incubated with $FeCl_3$ (12.5, 25, or 50 μM) and then added to the gametocyte culture. Gametocytes were cultured with the FBS0701/$FeCl_3$ mixture for 24 hrs, fed to mosquitoes and oocyst numbers were determined 7 days post infection. The results showed that when the drug is pre incubated with $FeCl_3$ the oocyst number is considerably higher compared to the FBS0701 treated culture. Individual iron supplementations showed negative effects on oocyst number although they were not as high in numbers compared to their respective counterparts in the combinations Fe-FBS0701 (Figure 4.4A). As can be seen on figure 4.4B, FBS0701 at 25 μM induced an oocyst inhibition higher than 80% whereas FBS0701-Fe showed 0% in oocyst inhibition relative to the effect seen with FBS0701 25μM (Figure 4.4B). The reduction in prevalence was around 50% for cultures treated with FBS0701 whereas those treated with FBS0701-Fe or iron alone reached a prevalence reduction no higher than 20% (Figure 4.4C).



FBS (µM)	0	25	25	25	25	0	0	0
Fe (µM)	0	0	12.5	25	50	12.5	25	50
Period (hrs)	24	24	24	24	24	24	24	24
Mean oocyst	14.3	0.16	17.2	25.7	19.3	10.5	11.8	11.6
% inhibition	-	98.9	0	0	0	26.9	17.8	19.2
Prevalence	92.5	12.3	88.9	95.1	96.9	84.6	97.4	92.9
N	67	57	54	61	65	52	39	42
P-value	-	< 0.001	NS	< 0.001	NS	NS	NS	NS

Figure 4.4: Iron restores infectivity to *P. falciparum* gametocytes incubated with FBS0701. To determine if the inhibitory effect of FBS0701 (or FBS) on gametocytes infectivity was due to iron chelation, FBS0701 was pre incubated with different concentrations of Fe before adding it to the gametocyte culture. Gametocytes were incubated with FBS0701/Fe for 24 hrs and then fed to *An. gambiae* mosquitoes. Oocyst numbers were determined in mosquito midguts 7 days post infection. **A.** Representative experiment. N= number of mosquitoes. Percent inhibition = [(mean oocyst # of control mosquitoes - mean oocyst # of experimental mosquitoes) / mean oocyst # of control mosquitoes] x 100. Prevalence = percentage of infected mosquitoes, Percentage reduction in prevalence = [(prevalence of control mosquitoes – prevalence of experimental mosquitoes) / prevalence of control mosquitoes] x 100. Significance of differences between the mean oocyst number of FBS0701 treatments compared to blank control were determined by One way Anova with Bonferroni's Multiple Comparison Test. NS= not significant. **B.** Percent inhibition of oocyst formation. Bars represent the average % inhibition of oocyst formation from independent experiments shown in Table A3 (pooled data). Error bars represent the standard error of the mean. **C**. Percent reduction in prevalence. Bars represent the average % reduction in prevalence (% infected mosquitoes) compared to the blank control, from independent experiments shown in Table A3 (pooled data). Error bars represent the standard error of the mean.

4.4.5 Mature *P. falciparum* gametocytes treated with FBS0701 show decreased numbers of exflagellations

The reduction in oocyst numbers detected after gametocyte treatment with FBS0701 could result from a failure of gametocyte activation into gametes or a failure of development into the subsequent mosquito midgut stages (zygotes, ookinetes and oocysts). Gametocyte activation into gametes is the first step of parasite development in the mosquito midgut lumen and can be measured by quantifying the number of male gamete exflagellations. To determine if FBS0701 treatment inhibits gametocyte activation, male gamete exflagellations were measured in gametocyte cultures incubated in the presence (25 μM) or absence of FBS0701 for 48 hrs. We did not find exflagellations on the FBS0701 treated gametocyte culture (Figure 4.5) compared to untreated control group.

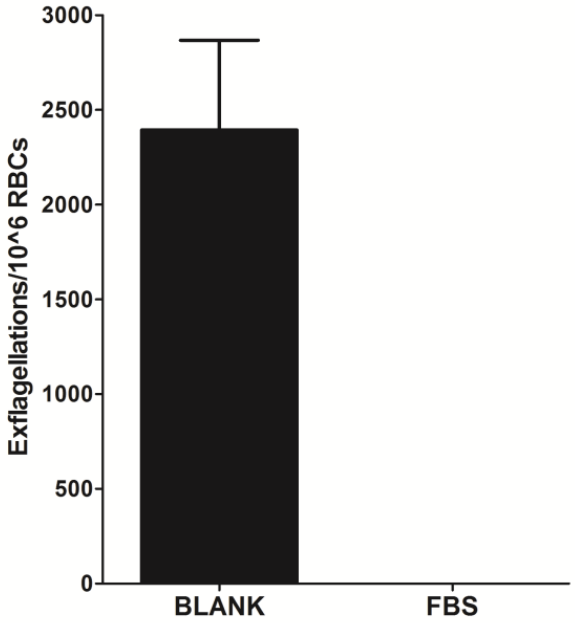


Figure 4.5. FBS0701 at 25 µM for 48 h decreases (inhibits) exflagellation centers. FBS0701 (or FBS) treated mature gametocytes were activated with exflagellation media containing 1 uM xanthurenic acid and incubated at room temperature. Exflagellation centers were counted 15 min post activation and observed at 40 X (Olympus BX53 microscope) in duplicates. Results are presented as the numbers of exflagellation centers per 10^6 erythrocytes in the treated and non treated cultures.

4.4.6 FBS0701 kills mature *P. falciparum* gametocytes

A reduction in male exflagellations after treatment with FBS0701 could result from inhibition of gamete activation or gametocyte killing induced by the drug. To test this hypothesis, the membrane impermeant propidium iodide (PI) fluorescent molecule was used for life/dead staining of gametocytes incubated with FBS0701. Only dead cells stain with PI as their membrane become permeable and the dye gain access to the DNA and RNA of the cell. A significant reduction in live gametocytes was observed for gametocytes treated with 25 μM for 48 hrs of FBS0701 (95 live and 5 dead) compared to the untreated culture (101 live and 0 dead) (Figure. 4.6).

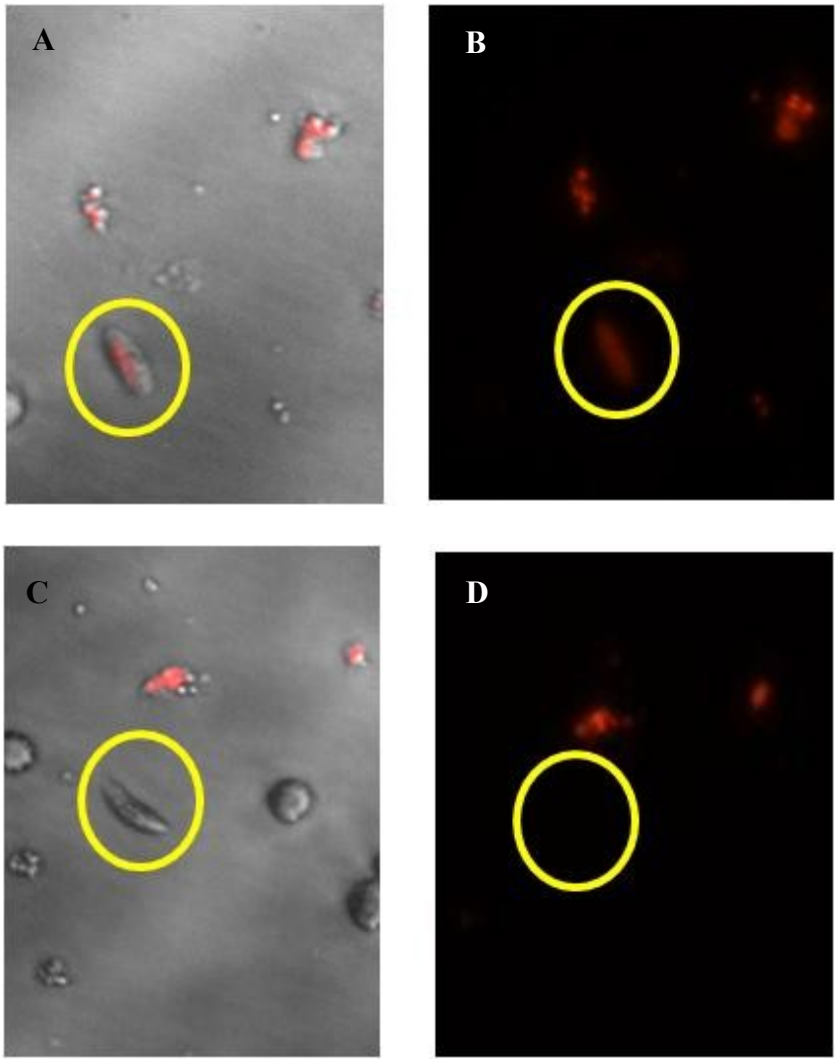


Figure 4.6. FBS0701 at 25 µM for 48 h kills fifteen day old *P. falciparum* gametocytes. FBS0701 treated gametocytes were incubated with 10 µl propidium iodide (PI) (100 ug/ml) and incubated for 10 min. After pelleting, the culture was resuspended in 1X PBS. Fluorescent microscopy imaging showing representative gametocytes from 25 µM FBS0701 treated (A-B) and non treated cultures (C-D) after 48 hours. Gametocytes are observed within the yellow circles. **A and C**. Pictures on phase 3 (red). **B and D**. Merged pictures of phase 3 and red. Scale bar 16 µm.

4.5 DISCUSSION

As discussed in other studies the antimalarial properties of iron chelators are known[25]. The new iron chelator FBS0701 was found to have antimalarial properties in the *Plasmodium* blood stages *in vitro* and *in vivo*[158]. FBS0701 was not able to affect late gametocyte stages when examined by blood film. *Anopheles gambiae* mosquitoes conserve iron absorption proteins and intracellular iron utilization proteins identical to the proteins in mammals[185]. Once iron is ingested in the blood meal it has to be transported to the ovaries for egg development. For the vector host, iron is an important element for producing offspring but not for individual survival. After all, male and female mosquitoes can survive individually mainly from a sugar source. From this point, is it understandable that the FBS0701 action might be null. The parasite would readapt its metabolism in the new host and take advantage of the nutrients and elements abundant and/or important for the host itself.

In a previous publication, FBS0701 was found to be effective predominantly on early stage gametocytes (around 90% inhibition) versus late stage gametocytes (around 50% inhibition) under the same dose of 100 μM[158]. Here we demonstrated that even when late stage gametocytes are treated with FBS0701 we could induce a significant decrease in oocysts after an artificial mosquito feeding. Now with the evidence of a partial inhibition on late stages from the past study by Ferrer *et al.*[158] and our results on oocyst reduction we considered two possibilities: remaining gametocytes after treatment could have their exflagellation impaired or they could be already dead before the membrane feeding. We confirmed that FBS0701 treated gametocytes had lower number

of exflagellation centers but this is the result of a smaller living population in the treated cultures. The propidium iodide confirmed that those gametocytes present counted by light microscopy before the membrane feeding were already dead.

In the study by Ferrer *et al*. [158], it was confirmed that FBS0701 could chelate and remove intracellular iron, whereas the antimalarial deferoxamine, a lipophobic iron chelator, may not access and chelate intracellular iron with effectiveness. This would explain the higher inhibition of oocyst formation under FBS0701 treatment compared to deferoxamine.

We were able to confirm that the decrease in oocyst numbers was because of FBS0701 action, when we blocked the drug with ferric chloride the chelating activity was suppressed allowing more oocysts to be formed in the mosquito midguts.

The mitochondria of *Plasmodium* asexual stages and early gametocytes have few cristae. Stages III, IV and V macrogametocyte show an increase in cristae mitochondria but microgametes have fewer mitochondria[178,186]. There is also an increased expression of the heme binding protein cytochrome b in sexual stages[181,187]. The tricarboxilic acid would be more active during sexual stages to produce succinyl Co-A for heme biosynthesis. However, it is also suggested that sexual parasites could have an underdeveloped metabolically active TCA cycle for respiration[178,179].

Besides energy production, the electron transport system can also be used for pyrimidine biosynthesis. It is suggested that the mitochondrial activity seen in the sexual stages is meant to fulfill this goal. De novo pyrimidine synthesis in early gametocytes

would be enough for the sustainment of the maturation process[168,188]. So the question is how can an iron chelator induce parasite death by removing an element that apparently is not indispensible especially for late stage gametocytes? Early gametocytes do not replicate their DNA so we discard the possibility of FBS0701 interfering with DNA replication by depriving ribonucleotide reductase from iron. We hypothesize that FBS0701 can exert a toxic mechanism or interfere with the energy production system which can still work on gametocytes; after all, protein components of the electron transport system bind iron and this metal may not be that indispensable for those proteins.

CHAPTER 5

GENERAL DISCUSSION

Iron is important for all living organisms and we demonstrated here that it is also important for *Plasmodium* development. Our first purpose was to understand the importance of iron compartmentalization and bioavailability from the host cells for the blood and liver stages of the parasite life cycle. Second, evaluate the parasitic need for bioavailable iron within the hepcidin – iron – infection axis in a context of iron deficient anemia and non anemia and iron supplementations.

The fight against malaria is difficult because of the development of resistant *Plasmodium* strains. Identification of a target not prone to amino acid mutation is important. Iron chelators have proven to be a good alternative to circumvent mutations on their non mutable drug target-iron. However, their use has been limited because of unsuitable pharmacokinetic properties. FBS0701 was designed to treat transfusional iron overload and when we obtained the drug for our studies it was under current Phase 2 human studies. Before our research began, we were aware of the advantages of this drug: its favorable absorption, high affinity for ferric iron and pharmacokinetics properties all of which allowed single daily dose to be effective[109,110].

We proved that FBS0701 can effectively sequester and remove intracellular iron from the erythrocyte consequently inhibiting the development of the parasite on blood stages. The fact that FBS0701 also affects the liver stage, led us think that FBS0701 also deprived intracellular hepatic iron. Iron removal is a distinctive characteristic compared to other drugs since it may not only be the cause of its action but also allows the drug to be effective even after its clearance from plasma (post-drug effect). Early studies suggested that iron chelators interfere especially on replicating stages of *Plasmodium*

(interfering with the DNA replication)[189,184,98]. According to this idea, iron chelators should have a higher effect on the late trophozoites. We found that upon continuous cultures starting at specific parasite stages, the IC_{50} for FBS0701 was 6 μM for rings and trophozoites, although 15 μM for schizonts which is past point of DNA replication. Chelation by FBS0701 is stage and concentration dependent. On our initial studies on gametocytes we observed that early stages were more affected (decreased numbers) compared to late stages that were morphologically intact by Giemsa film. Although early gametocytes do not replicate DNA, they are very similar metabolically to asexual stages[132]. Later we studied the effect of FBS0701 on 15 –day old *P. falciparum* gametocytes and evaluated the effect by the numbers of oocyst forming in mosquito midguts after membrane feeding. We found a decrease in the number and prevalence of oocysts after drug treatment of gametocytes. We even found that FBS0701 was more effective than DFO. This finding led us reconsider if FBS0701 was really inhibiting the DNA replication only. After staining treated gametocytes with propidium iodide we confirmed that parasites were killed by the drug and therefore reduced effective number of oocysts to near zero with microM concentrations of FBS0701. By microscopy, the morphology of gametocytes is intact. We think that the mechanism of action of iron chelators may be related to interference with DNA replication in asexual stages and even on hepatic stages but it could change to or simultaneously be toxic by interfering with the cellular energy system. Sexual stages have increased expression of proteins involved electron transport reactions. Cytochromes in the electron transport chain contain a heme prosthetic group which binds iron[25].

Our studies suggest the FBS0701 can be used in combinatory therapy with chloroquine, quinine (both found in vitro) and primaquine (found in vivo). Unfortunately, we found interference with artemisinin. Because the drug has a prolonged effect even after being fully metabolized in the liver, FBS0701 could be used as prophylactic or in combination with other antimalarials during the treatment of a current infection. FBS0701 may perturb an iron compartment on the circulating erythrocyte that may not be restored until the formation of new red blood cells. We demonstrated that we can have complete cure on mice receiving a single oral dose one day post infection. Some concern is raised about the use of iron chelators to treat malaria since most populations at risk of infection suffer from iron deficient anemia; this concern is even higher for anemic children. Based on the pharmacodynamics of the drug, FBS0701 at 32 mg/kg would not remove more than 20 mg of iron from the host over 24 h, considering that normal iron body stores range around 2-6 g. This amount should not affect total iron status in a short 3-5 day treatment regimen for malaria.

One advantage that we found about FBS0701 as antimalarial is that it targets more than one single stage of malaria. This makes the iron chelator attractive for elimination strategies. Although we proved that it does not interfere directly with mosquito stages, but may contribute to block the transmission of viable gametocytes to the mosquito. An additional study would be to test the effect of parasite development on mosquito stages after feeding the vectors on infected FBS0701 treated mice. Also, if resistance was to emerge, the mechanism or resistance would likely be different from other antimalarial drug resistance mechanisms. The action of FBS0701 is mainly due to sequestration of iron.

Our studies on iron diets, phlebotomized anemic mice, transgenic anemic mice, and non-anemic mice evaluated parasite development on different settings of hepatic iron. We confirmed the importance of iron compartmentalization with the hepcidin transgenic mice (low iron on hepatocytes) and hemoglobin deficient mice (or hbd, defect on erythroid progenitor cells but with unchanged hepatocyte iron stores). The hepcidin transgenic mice developed more pronounced liver stage malaria compared to the hbd mice. There are some questions that we have not addressed here: a) the blood stage infection on transgenic mice, though we speculate that malaria liver stages would be more pronounced compared to the blood stage and b) the effect of iron supplementation on hbd at both the hepatic and blood stages. We would expect to see a protection from severe disease caused by a blood stage and similar effects on the liver stages as the ones we have just reported in our study, in other words, high levels of parasite loads in the liver.

There are contradictory results about the negative or null effect of iron supplementation on malaria risks[118,119,120,143,146]. Our work tried to replicate the Pemba trial although we did not give folate supplementation simultaneously as on the African study[112]. We used the same hematologic biomarkers as on Pemba: hemoglobin and ZnPPIX , though we are aware that sometimes the use of different parameters, e.g. ferritin saturation, has led to contradictory results[190]. However, we obtained similar results to the Pemba trial. Our findings tell us that iron replete individuals after iron supplementation increase their hepatic parasite load. Also, in the hepcidin -iron – infection axis the dominant regulator may vary with time. Hepcidin could predominate at the beginning but iron would be dominant regulator over time. We are also considering a

change on hepatocyte ferroportin responsiveness in the context of anemia and iron supplementation. In areas where malaria is endemic and people are under constant infections or superinfections, malaria contributes to anemia, and hepcidin can also be increased by malaria infection by unknown mechanisms. These contexts refer to blood stages of malaria while we only tested the effect on liver stage. It would be interesting for future research to repeat the experiment adding an antimalarial such as primaquine along with iron supplementation and see the effect on hepatic parasite load. A previous study found that an ongoing blood stage infection treated with antimalarials did not preclude the hepatic development of a secondary infection in the context of a superinfection[9]. This was the result of the reduction in hepcidin expression because the blood stage parasitemia diminished or was cured. On a repetition of our experiment we would expect to see no difference in the parasite load between anemic and non-anemic mice under an ongoing antimalarial treatment and iron supplementation. We only tested the effect with one type of iron supplementation (iron diet) but future studies could also explore the difference in type or iron supplementation: e.g. intraperitonael iron dextran and blood transfusion. Blood stage parasitemia interferes with the use of iron from the host. We believe that our findings support the idea that iron status influences malaria risks and that iron supplementation can be positive for parasite development in the liver (site of the beginning of the infection); especially when the liver iron stores are replete. Policies of iron supplementation in malaria endemic and iron deficient areas should include antimalarial treatment and surveillance simultaneously. However, it is important for the iron supplementation policies to consider that malaria endemic areas overlap with iron deficiency anemia, and also overlap with other infectious diseases. Iron is important

for all living organisms, and the availability of the metal can regulate the susceptibility to other non-malaria infectious diseases. Upon iron supplementation studies have shown to increase cases of malaria, brucellosis and schistosomiasis[119]. We have not tested FBS0701 on other organisms but it could also be useful to target more than one microorganism at a time.

In summary (diagram below), we have confirmed that *Plasmodium* development requires iron. We believe it can be used for DNA replication or the function of electron transporter chain for energy production. The new iron chelator FBS0701 can sequester and remove intercellular iron from erythrocytes decreasing the development of asexual and sexual stages. Similar mechanisms may occur in the hepatic stage; we observed a reduction of parasite load in the liver upon murine infection. FBS0701 could even have the potential as a transmission blocking agent by killing mature stage gametocytes. FBS0701 could be used as part of a combination therapy with drugs that target any stage of the parasite except with artemisinin. We have also confirmed that iron supplementation increase parasite loads on hepatic stages in the context of non-anemia. Policies on iron supplementation in malaria endemic areas should be implemented along with a surveillance programs and antimalarial treatments.

Early sexual stage (early gametocytes) numbers reduced by:

- Iron chelator FBS0701

Late sexual stage (late gametocytes) killed by:

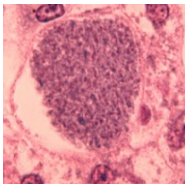
- Iron chelator FBS0701

Mosquito midgut stage not affected by iron chelator FBS0701

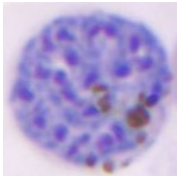
Mosquito stage

Sexual stage

Liver stage



Erythrocytic stage



Liver stage decreased by:

- Iron chelator FBS0701
- Synergistic action between FBS0701 and primaquine
- Redistribution of iron (compartmentalization) after hepcidin expression
- High levels of hepcidin

Liver stage increased by:

- Low levels of hepcidin
- Iron supplementation in iron replete mice (non-anemic, non iron deficient)
- Predominance of iron supplementation over a negative hepcidin effect

Erythrocytic stage inhibited by:

- Iron chelator FBS0701

FBS0701 effect on erythrocytic stage:

- IC_{50} stage specific and lower compared to deferoxamine and deferiprone
- Additive action with chloroquine and quinine
- Confines infection to reticulocytes in a *P. yoellii* infection and cures infected mice
- Chelates and removes intracellular iron

APPENDIX:

SUPPLEMENTARY DATA

Experiment	Treatment	Period (hrs)	uM	N	Prevalence	Mean oocyst # (Range)	% inhibition	P-value
1	Blank	24	0	35	97.1	90.6 (0-302)	-	-
	FBS	24	12.5	29	100	85.0 (23-204)	6.19	NS
	FBS	24	25	40	87.5	17.8 (0-48)	80.4	<0.001
	Blank	48	0	35	94.3	41.5 (0-129)	-	-
	FBS	48	12.5	32	87.5	16.2 (0-50)	61	<0.001
	FBS	48	25	28	10.7	0.11 (0-1)	99.7	<0.001
	Blank	72	0	30	63.3	35.4 (0-116)	-	-
	FBS	72	12.5	27	14.8	0.19 (0-2)	99.5	<0.001
	FBS	72	25	18	0	0 (0)	100	<0.001
2	Blank	24	0	45	93.3	34.6 (0-116)	-	-
	FBS	24	25	48	52.1	1.3 (0-9)	96.2	<0.001
	FBS	24	50	45	26.7	0.73 (0-9)	97.9	<0.001
	Blank	48	0	40	87.5	32.1 (0-107)	-	-
	FBS	48	25	41	17.1	0.17 (0-1)	99.5	<0.001
	FBS	48	50	45	6.7	0.07 (0-1)	99.8	<0.001
	Blank	72	0	39	61.5	10.1 (0-56)	-	-
	FBS	72	25	42	4.8	0.07 (0-2)	99.3	<0.001
	FBS	72	50	37	5.4	0.05 (0-1)	99.5	<0.001
3	Blank	48	0	39	79.5	48.2 (0-172)	-	-
	FBS	48	12.5	40	82.5	23.0 (0-60)	52.3	<0.001
	FBS	48	25	49	8.2	0.14 (0-4)	99.7	<0.001
	Blank	72	0	38	76.3	29.4 (0-104)	-	-
	FBS	72	12.5	43	44.2	1.28 (0-10)	95.6	<0.001
4	Blank	24	0	29	67.1	3.1 (0-16)	-	-
	FBS	24	25	32	9.4	0.13 (0-2)	96	<0.001
	FBS	24	50	28	10.7	0.11 (0-1)	96.6	<0.001

Table A1: Effect of incubation of *P. falciparum* mature gametocytes with FBS0701 on mosquito stage development. *P. falciparum* mature gametocytes were incubated with different FBS (FBS0701) concentrations for 14, 48 and 72 hrs. and then fed to *An. gambiae* female mosquitoes. Oocyst numbers were determined on mosquito midguts dissected 7 days post infection. N= number of mosquitoes, prevalence= % of infected mosquitoes, percent inhibition = ([mean or median oocyst # for control mosquitoes - mean or median oocyst # for experimental mosquitoes] / mean or median oocyst # for control mosquitoes) X 100. Statistical significance was determined by Mann Whitney U Test, a = 0.05. NS= not significant.

Experiment	Treatment	Period (hrs)	µM	N	Prevalence	Mean oocyst # (Range)	% inhibition	P-value
1	Blank	24	0	52	**75**	25.3 (0-115)	-	-
	DFO	24	12.5	45	**71.1**	17.6 (0-90)	**30.6**	NS
	DFO	24	25	38	**78**	18.1 (0-93)	**28.3**	NS
	Blank	48	0	43	**53.5**	24.1 (0-124)	-	-
	DFO	48	12.5	39	**61.5**	23.2 (0-89)	**3.7**	NS
	DFO	48	25	50	**42**	12.7 (0-98)	**47.2**	NS
	Blank	72	0	43	**55.8**	26.1 (0-112)	-	-
	DFO	72	12.5	45	**55.6**	20.6 (0-122)	**21.1**	NS
	DFO	72	25	39	**48.7**	10.7 (0-45)	**59**	NS
2	Blank	24	0	37	**78.4**	13.7 (0-66)	-	-
	DFO	24	12.5	45	**86.7**	9.36 (0-62)	**31.7**	NS
	DFO	24	25	42	**83.3**	14.1 (0-34)	**0**	NS
	Blank	48	0	50	**92**	7.94 (0-31)	-	-
	DFO	48	12.5	46	**73.9**	7.57 (0-37)	**4.72**	NS
	DFO	48	25	32	**65.6**	3.41 (0-18)	**57.1**	< 0.05
	Blank	72	0	32	**81.3**	9.06 (0-31)	-	-
	DFO	72	12.5	37	**59.5**	2.76 (0-16)	**69.6**	< 0.001
	DFO	72	25	42	**27.9**	0.72 (0-6)	**92**	< 0.001
3	Blank	24	0	22	**90.9**	18.5 (0-90)	-	-
	DFO	24	12.5	23	**95.7**	24.2 (0-62)	**0**	NS
	DFO	24	25	23	**82.6**	24.9 (0-97)	**0**	NS
	Blank	48	0	27	**85.2**	16.0 (0-53)	-	-
	DFO	48	12.5	26	**73.1**	15.9 (0-61)	**0.49**	NS
	DFO	48	25	24	**75**	13.8 (0-55)	**13.6**	NS

Table A2: Effect of incubation of *P. falciparum* mature gametocytes with DFO on mosquito stage development. *P. falciparum* mature gametocytes were incubated with different FBS (FBS0701) concentrations for 14, 48 and 72 hrs. and then fed to *An. gambiae* female mosquitoes. Oocyst numbers were determined on mosquito midguts dissected 7 days post infection. N= number of mosquitoes, prevalence= % of infected mosquitoes, percent inhibition = ([mean or median oocyst # for control mosquitoes - mean or median oocyst # for experimental mosquitoes] / mean or median oocyst # for control mosquitoes) X 100. Statistical significance was determined by Mann Whitney U Test, a = 0.05. NS= not significant.

Experiment	Treatment	Period (hrs)	µM	N	Prevalence	Mean oocyst # (Range)	% inhibition	P-value
1	Blank	24	0/0	41	87.8	34.9 (0-127)	-	-
	FBS/Fe	24	25/12.5	37	97.3	57.8 (0-115)	0	< 0.01
	FBS/Fe	24	25/25	48	97.3	44.3 (0-131)	0	< 0.05
	FBS/Fe	24	25/50	55	98.2	40.1 (0-102)	0	NS
	Fe	24	0/12.5	40	97.5	38.0 (0-120)	0	NS
	Fe	24	0/25	39	92.3	20.9 (0-65)	40.2	NS
	Fe	24	0/50	40	85	17.5 (0-70)	50	NS
2	Blank	24	0/0	67	92.5	14.3 (0-36)	-	-
	FBS	24	25/0	57	12.3	0.16 (0-3)	98.9	< 0.001
	FBS/Fe	24	25/12.5	54	88.9	17.2 (0-53)	0	NS
	FBS/Fe	24	25/25	61	95.1	25.7 (0-68)	0	< 0.001
	FBS/Fe	24	25/50	65	96.9	19.3 (0-92)	0	NS
	Fe	24	0/12.5	52	84.6	10.5 (0-49)	26.9	NS
	Fe	24	0/25	39	97.4	11.8 (0-28)	17.8	NS
	Fe	24	0/50	42	92.9	11.6 (0-41)	19.2	NS
3	Blank	24	0/0	43	88.4	54.7 (0-197)	-	-
	FBS	24	25/0	39	74.4	9.05 (0-29)	83.4	< 0.01
	FBS/Fe	24	25/12.5	42	83.3	55.6 (0-241)	0	NS
	FBS/Fe	24	25/25	43	86.1	65.2 (0-283)	0	NS
	FBS/Fe	24	25/50	39	79.5	71.6 (0-227)	0	NS
	Fe	24	0/12.5	33	84.9	23.6 (0-118)	56.8	NS
	Fe	24	0/25	39	84.6	31.1 (0-161)	43.2	NS
	Fe	24	0/50	33	84.9	46.3 (0-176)	15.2	NS

Table A3: Effect of incubation of *P. falciparum* mature gametocytes with FBS0701 and Fe on mosquito stage development. FBS (FBS0701) was incubated with different concentrations of Fe before adding it to the *P. falciparum* gametocytes. Gametocytes were incubated with FBS and or Fe for 24 hrs and then fed to *An. gambiae* female mosquitoes. Oocyst numbers were determined on mosquito midguts dissected 7 days post infection. N= number of mosquitoes, prevalence= % of infected mosquitoes, percent inhibition = ([mean or median oocyst # for control mosquitoes - mean or median oocyst # for experimental mosquitoes] / mean or median oocyst # for control mosquitoes) X 100. Statistical significance was determined by Mann Whitney U Test, a = 0.05. NS= not significant.

Experiment	Treatment	uM	N	Prevalence	Mean oocyst # (Range)	% inhibition	P-value
1	Blank	0	38	**92.1**	34.6 (0-152)	-	-
	FBS	3.38	37	**100**	30.5 (0-116)	**11.8**	NS
	FBS	6.75	39	**87.2**	24.9 (0-139)	**28.0**	NS
	FBS	12.5	42	**97.6**	27.0 (0-149)	**22.0**	NS
	FBS	25	44	**93.2**	26.8 (0-117)	**22.7**	NS
	FBS	50	43	**76.7**	16.5 (0-69)	**52.4**	NS
	FBS	100	37	**94.6**	21.6 (0-116)	**37.4**	NS
2	Blank	0	13	**100**	73.0 (8-207)	-	-
	FBS	12.5	10	**100**	87.6 (25-192)	**0**	NS
	FBS	25	9	**100**	53.8 (11-95)	**26.3**	NS
	FBS	50	25	**92**	42.0 (0-90)	**42.5**	NS
	FBS	100	25	**96**	46.8 (0-95)	**35.8**	NS

Table A4: Effect of FBS0701 on *P. falciparum* sexual reproduction inside the mosquito midgut. *P. falciparum* gametocytes cultures were fed to *An. gambiae* mosquitoes in the presence of different concentrations of FBS (without previous preincubation with the drug). Oocyst numbers were determined on mosquito midguts dissected 7 days post infection. N= number of mosquitoes, prevalence= % of infected mosquitoes, percent inhibition = ([mean or median oocyst # for control mosquitoes - mean or median oocyst # for experimental mosquitoes] / mean or median oocyst # for control mosquitoes) X 100. Statistical significance was determined by Mann Whitney U Test, a = 0.05. NS= not significant.

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PATRICIA FERRER

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Curriculum vitae

EDUCATION

2009 – June 2014 **Doctor of Philosophy (PhD.),** Department of Molecular Microbiology and Immunology,
JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH – JHSPH (BALTIMORE - USA)
Dissertation: Modulation of host iron compartments critical to the malaria parasite development

Advisor: Dr. David Sullivan Jr.

2011 **Certificate in Vaccine Policy and Science**, Department of International Health, JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH – JHSPH (BALTIMORE - USA)

2006 **Bachelor of Sciences (B.Sc.),** Biology
UNIVERSIDAD PERUANA CAYETANO HEREDIA (LIMA - PERU)
Senior Thesis: Effects of metal ions in the reactivation of *Mycobacterium tuberculosis* pyrazinamidase enzyme

Advisor: Dr. Patricia Sheen

FELLOWSHIPS AND AWARDS

2012 - 2014 Pre - doctoral JHMRI Fellowship
JOHNS HOPKINS MALARIA RESEARCH INSTITUTE – JHMRI (BALTIMORE – USA)

2011 The Dr. Harry J. Lawler Award Fund
JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH (BALTIMORE – USA)

2010 The Dr. Harry J. Lawler Award Fund

JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH (BALTIMORE – USA)

2006 Contenta of Sciences Award
UNIVERSIDAD PERUANA CAYETANO HEREDIA (LIMA - PERU)

RESEARCH EXPERIENCE

2010 - 2014 **PhD candidate**, JOHNS HOPKINS MALARIA REASEARCH INSTITUTE, JOHNS HOPKINS SCHOOL BLOOMBERG SCHOOL OF PUBLIC HEALTH, Baltimore - USA

PROJECT: Characterization of the antimalarial pharmacodynamics of the novel iron chelator FBS0701.

GOALS:

1. To identify the FBS0701 effective dose, timing and duration of antimalarial action *in vivo* and the IC_{50} and mechanism of action *in vitro*.
2. To characterize the interaction of FBS0701 with other antimalarials on blood and hepatic stages *in vitro* and *in vivo.*
3. To test the activity of FBS0701 on mosquito stages previous treatment of *P. falciparum* gametocytes.

PERFORMED THE FOLLOWING ASSAYS:

- Drug sensitivity assay with SYBR Green.
- Giemsa staining and counting of *Plasmodium* infected erythrocytes by microscopy.
- Murine drug testing after oral gavage and intraperitoneal *Plasmodium* infected erythrocytes.
- Blood collection by murine cardiac puncture.
- Cloning of plasmids for Real Time PCR quantification.
- Murine liver harvest and homogenization of livers with TRizol.
- Phenol-chloroform RNA isolation and Real Time PCR

quantification of parasite infection and gene expression.

- Salivary gland dissection of *Plasmodium* infected mosquitoes and sporozoite infection by tail vein injection of mice.
- Mosquito membrane feeding after drug testing of *Plasmodium* gametocyte cultures.
- Midgut dissection of *Plasmodium* infected mosquitoes and counting of mercurochrome-stained oocysts by microscopy.
- Exflagellation assays and viability determination by propidium iodide staining of drug treated *Plasmodium* gametocytes.

2010 - 2014 **PhD candidate**, JOHNS HOPKINS MALARIA REASEARCH INSTITUTE, JOHNS HOPKINS SCHOOL BLOOMBERG SCHOOL OF PUBLIC HEALTH, Balimore - USA

PROJECT: Evaluation of the effect of low and rich iron diets on murine malaria infection during iron repletion with implications for human nutritional iron supplementation in malaria endemic areas.

GOALS:

1. To compare the effect of a low, normal and high iron diet given for two weeks versus six weeks on hepatic malaria infection in iron replete mice and iron deficient mice.
2. To evaluate the level of hepatic malaria infection on transgenic overexpressing hepcidin (Tg+) mice upon the addition of exogenous hepcidin and on hemoglobin deficient mice (hbd).
3. To correlate malaria hepatic outcomes with the level of the iron regulatory hormone hepcidin in genotypic anemic mice, anemic non-genotypic mice and iron replete mice.

PERFORMED THE FOLLOWING ASSAYS:

- Murine phlebotomy by facial bleeding and eye bleeding.
- Murine liver harvest and homogenization of livers with TRizol.

- Phenol-chloroform RNA isolation and Real Time PCR quantification of parasite infection and gene expression.
- Salivary gland dissection of *Plasmodium* infected mosquitoes and sporozoite infection by tail vein injection of mice.
- Determination of anemia and iron deficiency by: hemoglobin estimation with Drabkin solution, ZnPPIX/heme measurement and haematocrit quantification.
- Quantification of nonheme iron on tissue by the *bathophenanthroline method.*

2008 **Research Associate,** INFECTIOUS DISEASES LABORATORY – LID,

UNIVERSIDAD PERUANA CAYETANO HEREDIA, Lima - Peru

PROJECT: Determination of *pncA* mRNA expression level in MTB isolates trough real time PCR.

GOAL: To study the relationship between the expression levels of *pncA gene* and the PZA resistance levels and enzymatic efficiency in mutant strains.

Performed mRNA quantification through real time PCR.

2006 - 2008 **Research Associate,** INFECTIOUS DISEASES LABORATORY – LID,

UNIVERSIDAD PERUANA CAYETANO HEREDIA, Lima - Peru

NIH Grant to PS (5-R03-AI-067608-02). - "Towards tuberculosis control: pyrazinamide susceptibility and resistant mechanism"

PROJECT: Relationship between Pyrazinamidase susceptibility and

Pyrazinamidase kinetic parameters in *Mycobacterium tuberculosis*

GOAL: To study the relationship between the enzymatic activity of recombinant Pyrazinamidases mutants bearing a single amino acid substitution with the microbiological pyrazinamamide susceptibility in their respective clinical isolates and the location of the mutation.

PERFORMED THE FOLLOWING ASSAYS:

- Selection of *M. tuberculosis* sputum isolates through Wayne test.
- Cloning of the *M. tuberculosis pncA* gene from mutant strains as well as from the wild type H37Rv reference strain.
- Expression and purification of the recombinant PZAases using affinity chromatography and SDS PAGE visualization.
- Measurement of PZA susceptibility parameters of the clinical isolates using Bactec 460TB, culture MIC and Wayne activity
- Measurement of kinetic parameters using Wayne activity
- Protein concentration determination by Bradford assay.

2006-2008 **Research Associate,** INFECTIOUS DISEASES LABORATORY – LID,

UNIVERSIDAD PERUANA CAYETANO HEREDIA

NIH Grant to PS (5-R03-AI-067608-02). - "Towards tuberculosis control: pyrazinamide susceptibility and resistant mechanism"

PROJECT: Effect of metals ions on the reactivation of Pyrazinamidase enzyme in *Mycobacterium tuberculosis*

GOALS:

1. To standardize the chelation method to inactivate the enzyme.
2. To identify the optimal concentrations on ions, through titration, to reactivate the chelated H37Rv Pyrazinamidase.
3. To determine the recovery activity percentages of chelated

Pyrazinamidase with the optimal concentrations of the ions.

4. To assess the consequences of an ionic interaction on enzymatic activity.
5. To determine the variation of chelated Pyrazinamidase enzyme kinetic parameters on the presence of different ions independently.

PERFORMED THE FOLLOWING ASSAYS:

- Cloning of the *M. tuberculosis pncA* gene from mutant strains as well as from the wild type H37Rv reference strain.
- Expression and purification of the recombinant PZAases using affinity chromatography and SDS PAGE visualization.
- Measurement of kinetic parameters using Wayne activity.
- Inactivation of Pyrazinamidase by depletion of metals using EDTA.
- Protein concentration determination by Bradford assay.

2005-2006 **Research Associate,** MOLECULAR NEUROBIOLOGY LABORATORY – LID, UNIVERSIDAD PERUANA CAYETANO HEREDIA, Lima - Peru

PROJECT: Use of the lithium ratio as a biological marker to diagnose bipolar disorder

GOALS:

1. To use the lithium ratio as the gold standard for diagnosing bipolar disorder.
2. To determine if the RPMI medium could be used instead of the Adenosine containing medium in the lithium rate method.
3. To identify erythrocytes membrane transporters associated with lithium transport and correlate their function with bipolar disorder.

Performed the Lithium ratio protocol proposed by Dorus *et al.* and modified by L. Poletti and G. Poletti.

2005 **Research Assistant,** INTERNATIONAL POTATO CENTER (IPC),

Lima - Peru

PROJECT: Biological and molecular characterization of the geographical isolates of PoGV (Granulovirus)

GOAL: To characterize genomic polymorphisms of geographical isolations of PoGV.

PROJECT: Molecular characterization of *Bemisia tabaci* biotypes in Cañete valley.

GOALS:

1. To evaluate adult nymphs of whitefly to identify species by taxonomic key and molecular biology
2. To identify *Bemisia tabaci* (Gennadius) biotypes in order to know the biotype that prevails in Peru.

PROJECT: Diagnosis of granulosis virus through PCR.

GOAL: To develop a sensitive method based on PCR as a complement to the traditional techniques.

PERFORMED THE FOLLOWING ASSAYS IN THE THREE PROJECTS:

- Biological activity of the geographical isolations: lethal concentration, bioassays and breeding of plague insect.
- Viral kinetics: counting of the viral multiplication inside each larvae making use of the Neubauer hemocytometer.
- Molecular characterization:
  - Massive production of PoGV
  - Viral purification and DNA extraction
  - PCR using specific primers for apoptotic genes
  - Recovery of DNA fragments from agarose gels (Promega kit).
- Design of a specific primer for granulin gene: bioinformatic programs GeneFisher, ClustalX, Blast.

- Standardization of RAPDs to diagnose biotypes of whitefly.
- Other:
  - Genetic transformation (Genetic Resources area).
  - Serology: NCM-ELISA and DAS-ELISA to detect main viruses of plants (Virology area).

2004 **Research Assistant,** INTERNATIONAL POTATO CENTER (IPC), Lima - Peru

PROJECT: Molecular characterization through RAPD-PCR of 18 isolations of *Beauveria brongnartii, Paecelimices Spp* and *Beauveria bassiana* in order to control the weevil of Andean crops.

GOAL: To understand the variability of fungus collection at molecular level and to cluster this isolation.

PROJECT: Search for a new entomopathogen to control *Symmetrischema tangolias* in Bolivia and Peru valleys.

GOALS:

1. To collect/identify new viral agents for *S. tangolias* control in the field and storage in Peru and Bolivia.
2. To characterize the isolated viral pathogen through RFLP analysis.

PROJECT: Spatial distribution of *Bemisia tabaci* whitefly and *Bemisia afer* in Cañete valley.

GOALS:

1. To identify species of whitefly (Homoptera: Aleyrodidae).
2. To study the seasonal variation and dissemination of *B. tabaci* and *B. afer* in sweet potato fields in Cañete valley.
3. To study the distribution and dissemination of whitefly species in Peruvian coast.

PERFORMED THE FOLLOWING ASSAYS IN THE THREE

PROJECTS:

- Preparation of stock solutions to use in molecular entomology.
- Multiplication of entomopathogenic fungi in PDA culture media.
- Use of RAPD-PCR to differentiate isolations of entomopathogenic fungi.
- Electrophoresis and silver staining of polyacrylamide gels.
- Electrophoresis and ethidium bromide staining of agarose gels.
- Bioassays and massive production of PoGV virus, isolated from *S.tangolias,* in *P. operculella* hostage.
- Counting of granulovirus using the Neubauer hemocytometer.
- Use of transmission electronic microscope (TEM) and scanning electronic microscope (SEM).
- Purification of PoGV virus from *S. tangolias.*
- DNA extraction from PoGV virus -*S. tangolias*.
- Characterization of different isolations of PoGV by RFLP analysis.
- Use of DNAstar program to design primers.
- Use of DAS-ELISA technique to diagnose viruses in insects.
- Whitefly mounting and use of taxonomic keys to identify species.

TEACHING EXPERIENCE

2013 **Teaching Assistant,** Department of Molecular Microbiology and Immunology, JOHNS HOPKINS SCHOOL OF PUBLIC HEALTH (Baltimore – USA)

Course title: Malariology

Communicated with students, graded assignments and exams; 1 term. (Dr. David Sullivan)

2006 **Teaching Assistant**, Department of Biochemistry, School of Medicine, UNIVERSIDAD PERUANA CAYETANO HEREDIA (Lima – Peru)

Course title: General Intermediate Biochemistry
Communicated with students, graded assignments and exams, conducted in-class tutoring and facilitated discussion group sessions; 1 term.

PUBLICATIONS

ARTICLES:

2014 **Ferrer, P**., Vega-Rodriguez, J., Jacobs-Lorena, M., Sullivan, DJ. [Effect of antimalarial iron chelator FBS0701 on *Plasmodium falciparum* mosquito stages]. To be submitted to *Antimicrobial Agents and Chemotherapy*

2014 **Ferrer, P**., Castillo, R., Roy, C., Sullivan, DJ. [Dynamics of *Plasmodium* liver stage, hepcidin and iron in iron deficient mice]. To be submitted to *Infection and Immunity*

2012 **Ferrer, P**., Tripathi, AK., Clark, MA., Hand, CC., Rienhoff, HY, Sullivan, DJ.

[Antimalarial Iron Chelator, FBS0701, Shows Asexual and Gametocyte *Plasmodium falciparum* Activity and Single Oral Dose Cure in a Murine Malaria Model]. *PLoS One*. 7(5):e37171

2012 Sheen, P., **Ferrer, P**., Gilman, R., Christiansen, G., Gutiérrez, A., Sotelo., Evangelista, W., Fuentes, P., Rueda, D., Flores, M., Olivera, P., Solis, J., Pesaresi, A., Lamba, D., Zimic, M. [Role of Metal Ions on the Activity of Mycobacterium tuberculosis Pyrazinamidase]. *American Journal of Tropical Medicine & Hygiene.* 87(1):153-61

2009 Sheen, P., **Ferrer, P**., López, J., Gilman, R., Fuentes, P., Valencia, E., Zimic, M. [Relationship between Pyrazinamide susceptibility and Pyrazinamidase kinetic parameters in *Mycobacterium tuberculosis*]. *TUBERCULOSIS (Eding).* 89(2):109-13

POSTERS:

2013 **Presenter**

Ferrer, P., Castillo, R., Roy, C., Sullivan, D. [Iron compartmentalization regulates hepatic *Plasmodium* growth].

MOLECULAR PARASITOLOGY MEETING XXIV – MARINE BIOLOGICAL LABORATORY, FROM SEPTEMBER 8th TO 12th IN WOODS HOLE, MASSACHUSSETS, USA.

2012 **Presenter**

Ferrer,P., Tripathi, A., Clark, M., Hand, C., Rienhoff, H., Sullivan, D.[Antimalarial iron chelator, FBS0701, shows asexual and gametocyte *Plasmodium falciparum* activity and single oral dose cure in murine malaria model].

MOLECULAR PARASITOLOGY MEETING XXIII– MARINE BIOLOGICAL LABORATORY, FROM SEPTEMBER 22nd TO 26th IN WOODS HOLE, MASSACHUSSETS, USA.

2011 **Presenter**

Tripathi, A., **Ferrer, P**., Rienhoff, H., Sullivan, D. [Single oral dose cure of lethal *P. yoelii* with a new iron chelator in human clinical trials for iron overload].

JOHNS HOPKINS MALARIA INSTITUTE AND THE RESEACRH ADVANCES IN MALARIA: RESISTANCE TO EXISTING DRUGS AND NEW DRUG DEVELOPMENT CONFERENCE, FROM JUNE 2nd TO 3rd IN TRES CANTOS, SPAIN.

2011 Baldeviano,G., Barin,J., Lei, W., Talor, M., **Ferrer, P**., Chen, P., Legault, J., Ong, S., Zheng, D., Bedja, D., Gabrielson, K., Chatzidimitriou, D., Rose, N., and Cihakova, D. [Eosinophils promote myocarditis and dilated cardiomyopathy by enhancing Th17 responses].

98th ANNUAL MEETING - THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS, FROM MAY 13th TO 17th IN SAN FRANCISCO, USA.

2008 Sheen, P., **Ferrer, P.,** Gilman, R., López, J., Fuentes, P., Christiansen, G., Sotelo, J., Evangelista, W., López, C., Valencia, E., Olivera, P., Solis,

J., Zimic., M. [Mycobacterium tuberculosis Pyrazinamidase: Association between enzymatic activity and the Pyrazinamide resistance level and characterization of the metal binding properties]. 43rd ANNUAL US-JAPAN TUBERCULOSIS AND LEPROSY CONFERENCE, FROM JULY 8th TO 10th IN BALTIMORE, USA.

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COMMUNITY SERVICE

2003 Collaboration in the systematization of technical work of the Payments for Environmental Services experience**s.** ASOCIACION QUECHUA-AYMARA, ANDES (CUSCO – PERU)

PROJECT: Ecosystem Sub-Global Assessment of the Vilcanota Sub-Region, developed by the Millennium Ecosystem Assessment Group.

PROJECT: Conservation strategy of the agricultural diversity of Santo Tomas river basin (Peru) by the Payments for Environmental Services (PES), promoted by the Universitat Autònoma de Barcelona, Spain.

RELEVANT PROFICIENCIES

2006 Advanced English Course. Included preparation for the "Certificate in Advanced English - CAE" from the University of Cambridge.

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1999 First Certificate in English (FCE). UNIVERSITY OF CAMBRIDGE-ESOL EXAMINATIONS